Cellular aging in cardiovascular diseases

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Content

Chapter 1

Cellular senescence and its role in aging

Introduction

Shakespeare formulated in Hamlet: "*Thou know'st 'tis common; all that lives must die*". Aging is one of the most universal aspects of life, a process that marks the end of all living organisms, but scientifically it remains an elusive process. As humans become older in our society, we are more and more faced with the aspects of aging problems. As we improve our understanding of aging it has become evident that aging already starts at young age. The accumulation of cellular damage already starts at an early age, and influences the process of aging for the long run. Cellular aging is also known as cellular senescence, a term derived from the Latin word *senex* that stands for old man or aged. This chapter discusses cellular senescence and the changes that occur at different levels during the development of senescence and aging.

Cellular aging

In 1881, August Weismann already suggested that death takes place because of worn-out tissue and that tissue cannot renew itself forever.[1, 2] In the 20th century this perspective on cellular aging was largely forgotten and the general assumption, which was strongly influenced by the work of the Nobel-prize winner Francis Carrel, was that all cells can divide indefinitely and that mortality in cell culture was a result of sub-optimal culture conditions.[3] In 1961, this dogma was overturned by the work of Leonard Hayflick and Paul Moorhead.[4] They showed that primary cells do stop dividing unrelated to cell culture conditions by an ingenious but simple experiment, they mixed young female and old male cells and after sub-culturing they showed that only female cells were present in the culture, providing evidence that the male cells could not have died because of culture conditions.[4] In their article, they describe different phases that can be distinguished during primary cell culture. The first phase is the primary culture phase, which is followed by a phase of constant growth and cell division. Eventually, cells reach as they described it "the phase III phenomenon" in which cell replication diminishes and eventually stops. This phase III became later known as "the Hayflick limit" or cellular senescence. The non-dividing cells remained viable for many weeks, but failed to grow despite the presence of growth factors and ample space.

The importance of cellular senescence in relation to aging of organisms became clear when it was shown that donor age of an organism influenced the proliferative capacity of cells.[5, 6] From the work of Hayflick and others it became clear that there might be an internal clock in all cells that determines mortality and aging.[7] It was in 1973 that the link between telomeres and the end-replication problem was suggested.[8, 9] The end-replication problem describes the predicament that during DNA replication the lacking strand of

the DNA cannot be completely replicated and therefore shortens at each mitotic cycle. A few years after the discovery of telomeres[10], and the enzyme telomerase[11] the importance of chromosomal ends in relation to the onset of senescence became clear.[12, 13] The role of telomere shortening and the role of the enzyme telomerase in aging are discussed in chapter 2 of this thesis.

Nowadays, we make a global distinction between two types of cellular senescence, both result in a comparable non-dividing end-point, but there is a difference on how senescence is originating. The non-dividing phase described by Hayflick where cells reach senescence after a number of population doublings is called replicative senescence. Not only old cells can become senescent, also relatively young cells can obtain a senescent phenotype. According to Harman's free radical theory of aging, in which he suggested that oxidative stress in the form of reactive oxygen species (ROS) and other redox reactions could negatively influence aging. This second form of senescence can be induced by different forms of cellular stress, such as UV or radioactive radiation[14], or oxidative stress[15], or exposure to increased levels of glucose.[16 17] The general accepted hypothesis for the onset of premature senescence is that, after sub-lethal doses of stress, a cell signaling cascade is activated that eventually results in a premature interphase G0 arrest – this process is termed stress induced premature senescence.

Morphological and molecular changes in senescence

Senescent cells undergo a very characteristic set of morphological changes that is easily recognizable in cell culture. As mentioned before senescent cells become arrested and can maintain this state in culture for a long period of time. In cell culture, senescent cells are relatively easy to recognize. The most obvious change is the increase of cell size, which is accompanied with a change in cytoskeletal proteins as actin, tubulin and vimentin.[18, 19] The cytoplasm becomes highly vacuolated and the nucleus is located centrally in senescent cells and in some senescent cells multi-nucleation occurs.

In vivo, senescent cells are not that easily recognizable. Senescence does not occur in cells that have lost the ability to proliferate, such as differentiated cardiac muscle and neuronal cells, but can originate in mitotic cells that have the potential to proliferate. As many cells in somatic tissue are in a quiescent state and hence do not divide for weeks to months, distinguishing senescent arrested cells from quiescent cells is difficult. However, also at the biochemical level changes occur in senescent cells and the increase of lysosomal β-galactosidase has been of great importance in senescence research.[20, 21] Dimri et al. described in 1995 that detection of β-galactosidase at a pH of 6 could be used as a marker

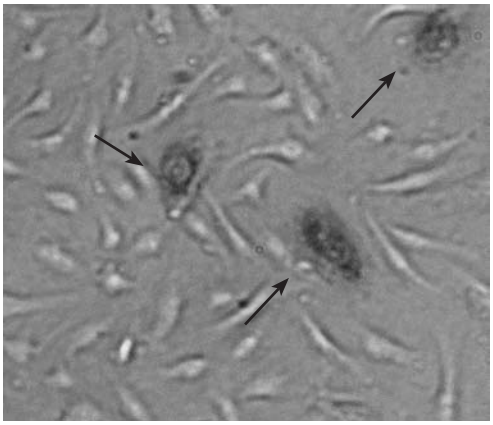

Figure 1. Senescent endothelial cells. Senescent HUVECs and neighboring non-senescent cells. Senescent cells stain positive for sa-β-gal, are larger, and can display an abnormal nuclear structure. Arrows indicate sa-β-gal positive cells.

for senescence *in vitro* and *in vivo*[20], and this staining is now widely used and has been termed senescence associated β-galactosidase (sa-β-gal) staining (Figure 1). Since then, there has been a lot of discussion on the relation of β-gal to cellular senescence and about the specificity of sa-β-gal.[22, 21, 23, 24] It was discovered that lysosomal β-gal is a side effect of aging and does not influence the onset of senescence.[24] We now know that sa-β-gal can be detected by sensitive HPLC methods in young and old cells[22], and also that serum deprived cells or cells that have reached confluence can stain positive for sa-β-gal.[23] Therefore, many researchers use multiple markers aside the sa-β-gal staining to establish senescence. Nevertheless, sa-β-gal staining remains the most used technique, since it can be easily conducted and is still accepted as a cellular marker for senescence *in vivo* and *in vitro*.

On the molecular level, there are two major pathways involved in the induction of senescence. Replicative, telomere dependent senescence and DNA damage induced senescence is associated with DNA damage signaling by ATM / ATR kinases that subsequently activates the tumor suppressor protein p53, which regulates the cyclin-dependent kinase inhibitor p21.[25] The second pathway is dependent on the activation $p38^{MAPK}$ followed by the activation of the cyclin-depend kinase inhibitor p16.[26] Activation of $p38^{MAPK}$ occurs after activation of the senescence by oxidative stress, and is also involved in protooncogene, induced senescence.[27] Interestingly, $p38^{MAPK}$ induced senescence can be telomere-dependent and telomere-independent.[26] There is also overlap with DNA damage induced senescence, as p53 can also become activated after $p38^{MAPK}$ induced senescence.[28] Activation of $P38^{MAPK}$ can increase intra-cellular levels of oxidative stress that results in p53 and p21 activation.[15, 29]

Both pathways eventually converge at the cell cycle regulator retinoblastoma protein (Rb) that in its hypophosphorylated active state inhibits progression to S-phase of the cell cycle (Figure 2). Although these pathways can induce senescence independently, there is overlap

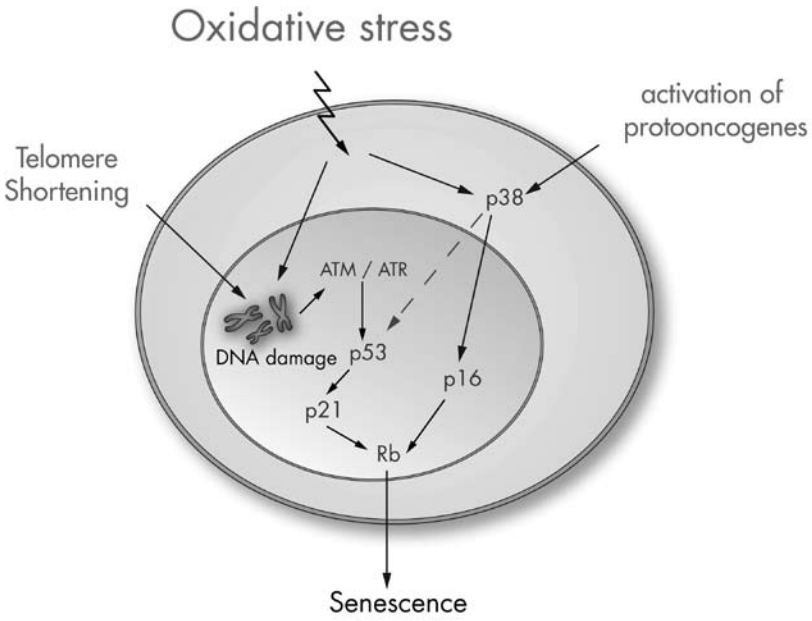

Figure 2. Pathways to senescence. Senescence is mediated by different pathways that converge on hypophosphorylation of Rb. Different forms of cellular stress can induce senescence either by activating p53/p21 or p38/p16 pathways.

between the pathways. The importance of a route can differ between cells and species. For instance, in human cells pRb can be activated independent of p53, through the upregulation of p16, while in mouse cells upregulation of p16 does not induce senescence.[30]

Cellular senescence and apoptosis

Senescence and apoptosis share common elements, both processes are a response to cellular stress and mark the end of a cell. But whereas in senescence a cell becomes arrested, apoptosis entails the complete destruction and subsequent elimination of stressed cells. How cells respond to stress can vary between cells and between cell types. Fibroblasts in the S-phase commit to apoptosis after sub-lethal doses of oxidative stress, whereas cells in the G1 and G2/M phase become senescent.[31] Exposure to a low dose of H_2O_2 induces senescence in fibroblasts, but exposure to higher concentrations results in a apoptotic outcome.[32] Stressors that induce senescence at a sub-lethal concentration can induce apoptosis at higher concentrations or prolonged exposure. It is still elusive, what determines the final fate

of a cell, but it probably depends on certain molecular thresholds. The tumor suppressor protein p53 is a critical component in controlling cellular activity. The protein can undergo a series of posttranscriptional changes, such as phophorylation and acetylation. In response to stress p53 becomes activated by posttranscriptional modifications and activates p21. The activation of p21 can result in senescence, but can also induce apoptosis depending on the activation of p16.[33] The exact mechanisms how p53 determines a cells fate, are still undetermined.

The occurrence and function of senescence in aging

The onset of senescence and the presence of senescent cells in primary cell culture is an accepted phenomenon, but it is still unclear what the role of senescence is in aging. Given that senescent cells do not proliferate, an anti-cancer mechanism has been suggested for this phenomenon.[34, 35] This idea is strengthened by the findings that oncogenes such as *RAS*, *RAF* and *PTEN* can induce senescence.[36, 37, 35] Recent data also suggest that senescence is *in vivo* an important tumor suppressor mechanism, in human and in mouse tissues.[38, 37, 39, 40] From an evolutionary point of view, a tumor suppressor function is not that peculiar. Selection for this trait in reproductive individuals may be beneficial for survival, it is at a later stage in life that the presence of senescent cells becomes less advantageous.

Advanced aging is associated with an increase in systemic levels of oxidative stress.[41, 42] In parallel there is a decrease in the antioxidant systems with aging.[43, 44] The hypothesis that senescence contributes to aging is therefore very tempting, but the concept is still under debate.[45, 46] With the discovery of more senescent markers, an increasing amount of evidence is gathered showing the presence of an increased number of senescent cells in aging tissue.[20, 47-51] In old baboons more than 15% of the dermal fibroblasts showed signs of senescence in the form of damaged telomeres, activated DNA damage response kinase ATM and increased levels of p16.[49, 50] An overall increase of the aging and DNA damage marker γH2AX was found in multiple organs such as the lung, spleen, dermis, liver and gut epithelium organs of aging mice.[51] In contrast, in these studies senescent markers were not detected in all organs, post mitotic tissue, such as skeletal and heart muscle was less affected by age.[50, 51] Increased levels of the senescence marker p16 were detected in human kidneys in relation to aging.[48] In diseased renal transplants, this marker was also increased suggesting that the presence of senescent cells in the diseased kidney and aging may share common pathways.[52] In line with this is the finding that senescence is also increased in the kidneys of patients with type II nephropathy.[53]

At the moment, it is unclear what happens to senescent cells in the body. There are differences in the susceptibility to apoptosis between different cell types. Senescent

fibroblasts are described to become less sensitive for apoptotic signaling[54], while aged endothelial cells seem to become more sensitive to apoptotic stimuli.[55] Another part of senescent biology that is still emerging, is the response of the immune system to senescent cells. In fibroblasts and endothelial cells, senescence is characterized by the upregulation of a gene pattern that is comparable to genes that are upregulated during inflammation. This is accompanied by an increase in inflammatory surface markers and cytokines.[56-58] These changes could possibly provoke clearance of these cells by the innate immune system[59], but this still needs proper conformation in different aging models, and further testing in human studies.

Vascular senescence

For vascular disease, advanced age is one of the major risk factors, even a higher risk than smoking, hypertension, diabetes or hypercholesterolemia.[60] As the vasculature is at the boundary of blood circulation and the underlying tissue, the vasculature is constantly exposed to a variety of stresses, such as hemodynamic stress, and oxidative stress in the form of reactive nitrogen species, ROS and different forms of oxidized lipids.[61, 62] The constant exposure to stress gives rise to the development of endothelial dysfunction, a pro-inflammatory state of the vasculature, in which the balance of vasoconstriction and vasodilatation is disrupted.[63] The occurrence of endothelial dysfunction is a hallmark of cardiovascular disease and occurs in patients with diabetes mellitus, hypertension, hypercholesterolemia, atherosclerosis and heart failure.[63] Therefore, improving endothelial function has the potential to decrease the risk for progression of cardiovascular disease and could potentially improve the quality of life of a large group of patients.

The occurrence of senescent cells in the vasculature has been most striking in atherosclerotic patients. Coronary artery explants, from ischemic heart disease patients, showed that the luminal surface of the vessel stained almost completely positive for sa-ß-gal, while internal mammary arteries of the patients were completely negative.[64] Histological examination of the positive vessels showed that it where predominantly endothelial cells that stained positive. In advanced atherosclerotic plaques senescent positive vascular smooth cells were also detected in the intima regions.[65] As senescent endothelial cells are more prone for apoptosis, this could *in vivo* result in denuded patches within the vasculature, exposing smooth muscle cells to the circulation and increase the incidence of atherosclerosis and thrombosis.[66]

Angiotensin II (Ang II) signaling is an important component of vascular stability and vascular function and seems to have an important influence on vascular aging as inhibition of Ang II can improve morbidity and mortality of cardiovascular disease.[67] Ang II can induce senescence in vascular smooth muscle cells via a p53/p21 dependent pathway that

is independent of telomere shortening[68, 69] and inhibition of the pathway can suppress senescence. Interestingly, loss of p21 ameliorates the induction of pro-inflammatory molecules by Ang II and prevents the development of atherosclerosis.[68, 70] Although the inactivation of p21 also influences the pro-inflammatory state and phagocytic activity of the macrophages, it is likely that the state of the macrophages also influences the development of atherosclerosis in these models.[70]

Endothelial senescence can be induced by diabetes mellitus, as has been shown in an animal model with streptozotocin, a broad spectrum antibiotic that has diabetogenic properties[71], in mice and rats.[16, 72] Senescent positive cells have also been detected in the aorta of Zucker diabetic fatty (ZDF) rats, an animal model that is used for studying diabetes mellitus and a model that develops endothelial dysfunction.[16, 73] Histological analysis showed an increase in cells staining positive for the senescent associated proteins p53, p21 and p16 in the vasculature of ZDF animals.[73]

Oxidative stress is a common denominator for the development of endothelial dysfunction.[61, 62] The vasculature can produce different forms of ROS, superoxide ($O_2^{-\bullet}$), nitric oxide (NO), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^{-\bullet}$), under normal and under stress conditions, such as inflammation or injury. ROS is produced by different enzymes, such as NADH oxidase, xanthine oxidase, cyclooxygenase, NO syntase (NOS), cytochrome P450 monooxygenase and by enzymes involved in the mitochondrial respiratory chain. Superoxide is relatively unstable and reacts (direct or by superoxide dismutase) to the more stable H_2O_2. Subsequently, H_2O_2 is converted to water and oxygen by catalase or gluthione peroxidase, during this reaction the highly active hydroxyl radical can be formed, which causes cellular and DNA damage.[74] The generation of ROS is essential for the regulation of cellular processes, and also influences vascular dilatation and constriction. Where an excess of ROS tips the balance to vasoconstriction, as ROS inhibits the three predominant vasodilator pathways; NO, prostacyclins and endothelial derived endothelium-derived hyperpolarizing factor (EDHF).[63] In the aging vasculature, the production of exo- and endogenous ROS increases, which negatively influences vasodilatation. NO is a crucial mediator of vasodilatation, and an excess of superoxide reduces the bioavailability of NO by generating the highly damaging agent peroxynitrite.[63] In addition, there is evidence that persisting oxidative stress will render the enzyme responsible for the generation of NO, endothelial NOS (eNOS), dysfunctional, such that it produces superoxide instead of NO.[75] Another factor that negatively influences the NO bioavailability is the down-regulation of eNOS in senescent cells.[64] Interestingly, inflammation plays a key role in the atherosclerosis and is suspected to be an important precursor of endothelial dysfunction.[63, 76] The onset of senescence coincides with the upregulation of inflammatory genes and proteins like ICAM-1, IL-6 and IL-8, which could intensify the pro-inflammatory state of the vasculature that is so conspicuous for endothelial dysfunction.[57]

The scope of this thesis

This thesis will discuss the effects of cellular aging, and the main focus will be at telomere biology and senescence in cardiovascular diseases. The first part of the thesis will discuss the effects of telomere shortening and the role of telomerase in aging associated diseases, with a focus on cardiovascular diseases. The main question discussed in this part is **how does telomere length and telomerase deficiency influence cardiovascular aging.**

Part II of the thesis will focus on the effects of stress induced cellular senescence on endothelial cells, in this part we will show how the activation of G-protein coupled receptors by specific hormones can activate cellular pathways that protect endothelial cells from oxidative stress induced senescence. The main question described in Part II is twofold: **Is it possible to prevent stress induced endothelial senescence, and what are the molecular mechanisms involved.**

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Chapter 2

Telomere and telomerase biology in healthy aging and disease

This chapter is based on:

Telomere biology in healthy aging and disease

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Telomere biology in cardiovascular disease: the TERC$^{-/-}$ mouse as a model for heart failure and ageing

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Abstract

Aging is a biological process that affects most cells, organisms and species. Telomeres have been postulated as a universal biological clock that shortens in parallel with aging in cells. Telomeres are located at the end of the chromosomes and consist of an evolutionary conserved repetitive nucleotide sequence ranging in length from a few hundred base pairs in yeast till several kilo base pairs in vertebrates. Telomeres associate with shelterin proteins and form a complex protecting the chromosomal deoxyribonucleic acid (DNA) from recognition by the DNA damage-repair system. Due to the "end-replication problem" telomeres shorten with each mitotic cycle resulting in cumulative telomere attrition during aging. When telomeres reach a critical length the cell will not further undergo cell divisions and become senescent or otherwise dysfunctional. Telomere shortening has not only been linked to aging but also to several age associated diseases, including tumorigenesis, coronary artery disease, and heart failure. In the current review, we will discuss the role of telomere biology in relation to aging and aging associated diseases.

"Death takes place because a worn-out tissue cannot forever renew itself, and because a capacity for increase by means of cell division is not everlasting but finite"

A. Weismann. Clarendon, Oxford 1881

A brief historical perspective

Telomeres are special deoxyribonucleic acid (DNA) structures that "cap" the ends of our chromosomes in conjunction with specialized proteins, the telomere-shelterin complex. This complex protects the chromosomes from erosion and end-to-end fusion. The term telomere originates from the Greek *telos*, which means "end" and *meros* which means "part". The existence of these end-parts of the chromosomes was first suggested in 1938 by Muller.[1] In 1961, Hayflick undermined a major paradigm of his time by providing convincing evidence that primary cells were not immortal, but could undergo only a limited number of cell divisions. This phenomenon, currently being referred to as the Hayflick limit[2], predicts the existence of an internal counting mechanism within the cell. Olovnikov, a Russian researcher, was the first who linked the end of the chromosomes to the cell cycle arrest described by Leonard Hayflick.[3] The term "end-replication problem" describes the effect that linear chromosomes cannot replicate their terminal ends of the chromosome and consequently shorten at each mitotic cycle. The first identification of the sequence of the terminal end of the chromosome (the telomere) in the *Tetrahymena* was discovered by Elizabeth Blackburn and Joseph Gal in 1978.[4] Ten years later, Robert Moyazis and colleagues revealed that the sequence of the human telomere consists of TTAGGG repeats.[5] Up to date, the sequence of many species and organisms have been established and we can conclude that the telomeres are an evolutionary well-conserved sequence (Table 1).[6] The next major breakthrough in telomere biology was the discovery of the reverse transcriptase telomerase by Carol Greider working as a postdoctoral student at the laboratory of Elizabeth Blackburn in 1985.[7] In contrast to DNA-polymerase, telomerase is capable of elongating the telomeres. Bypassing the end-replication problem for germ cells is essential to maintain telomere length for offspring. In 1997 Maria Blasco in the lab of Carol Greider created a telomerase deficient mouse, which had inactive telomerase and consequently reduced telomere length in each following generation.[8] The most striking characteristic of the telomeres in somatic cells is the shortening with age and in cell culture the telomere length is directly linked to the replicative capacity. In this review, we will discuss the function of the telomeres and will focus on the importance of telomere biology in normal aging and in pathology.

Table 1. Telomere length and telomere sequence in different species.

Species	Telomere length	Telomere sequence	Reference
Ciliates			
Protozoan (*T. thermophila*)	120 - 420 bp	T_2G_4	4
Yeast			
Baker's yeast (*S. cerevisiae*)	200 - 300 bp	$TG_{2-3}(TG)_{1-6}$	120
Vertebrates			
Humans	5 -15 kb	T_2AG_3	5
Mice	Up to 150 kb	T_2AG_3	21
Rats	20 -100 kb	T_2AG_3	22
Birds	5 – 20 kb	T_2AG_3	23
Invertebrate			
Ants	9-13 kb	T_2AG_2	121
Plants			
Thale cress (*A. thaliana*)	2 – 5 kb	T_3AG_3	122

Telomere; structure and T-loop

In vertebrates, the end of the chromosome, a G-rich strand, ends in a single strand extension of 75–200 bp, the G-tail (Figure 1). In the nonmitotic phase of the cell cycle this G-tail is shielded by a crucial so-called telomere shelterin complex in which the telomere binds internally by forming two internal loops, the D-loop and the T-loop.[9] The telomere shelterin complex is designed to protect the chromosomal ends from erosion and end-to-end fusion[10] and is formed by different proteins associated with the telomeres, such as telomeric repeat binding factor 1 (TRF1) and 2 (TRF2) that can bind to double stranded telomere DNA. Another telomere associated protein, protein protection of telomeres 1 (POT1), can bind directly to single stranded DNA, and it is suggested that POT1 binding to the 3'overhang is important for forming the D-loop. Other proteins involved in the shelterin complex that are recruited by TRF1 and TRF2 are repressor activator protein 1 (Rap1), TPP1, and TRF1-interacting nuclear factor 2 (TIN2) (Figure 2).[10] Telomere shortening will result in destabilization of the chromosomes and an inability to recruit the proteins of the shelterin complex. As a result, the T-loop cannot be formed as easily and the chromosome ends will be left uncapped. This is a situation that resembles double stranded DNA breaks, and presents a highly unstable cellular state that may lead to activation of the p53 or p16ink4a pathway and eventually can result in senescence or apoptosis.[11]

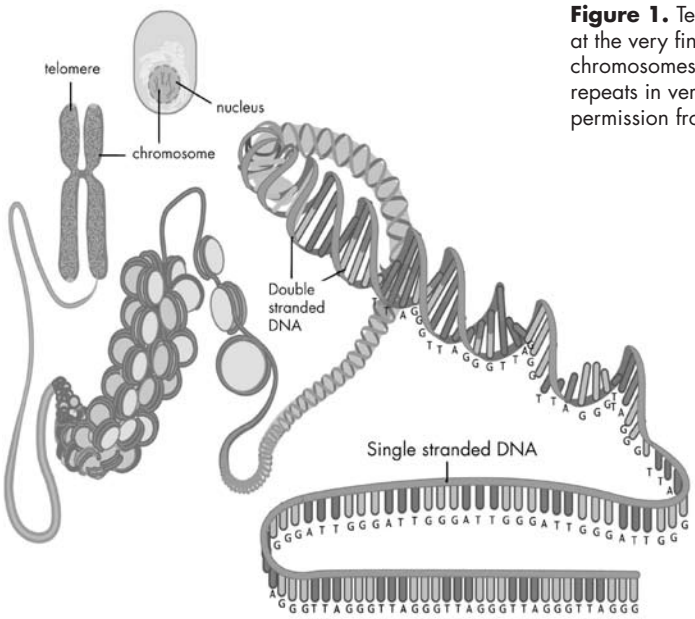

Figure 1. Telomeres are located at the very final ends of the linear chromosomes and consists of TTAGGG repeats in vertebrates. Reproduced with permission from[126].

Telomere length and aging

In contrast to the similarity of the sequence, the telomere length is highly variable among species, within species, within an organism, and even between chromosomes. In a study that evaluated telomere length in different organs from humans of different age, telomere length varied between 8 and 15 kbp and was highly variable between organs from one subject.[12] This may be explained by variable telomere attrition rate, in humans it is estimated that telomere length shortens 30–150 bp per replication cycle in fibroblasts and lymphocytes.[13, 14] There is a high amount of variance in telomere length in humans. Already at birth, remarkable differences in telomere length are observed. In addition, females have longer telomeres than men and in African Americans telomeres generally are longer than in White Americans.[15] As there is no gender difference at birth, it is most likely due to differences in environmental factors such as differences in estrogens levels.[16] Strikingly, macaques have approximately the same life span as humans but have longer telomeres in addition to a longer subtelomeric region.[17] Telomere attrition rate is not stable for each chromosome, in human cells the chromosomes 17p, 13p, and 19p have been identified as being shorter compared to the other chromosomes.[18, 19]

In humans, telomere length is measured extensively in leukocytes in relation to aging and various pathologies. Leukocyte telomere length obviously has the advantage of being

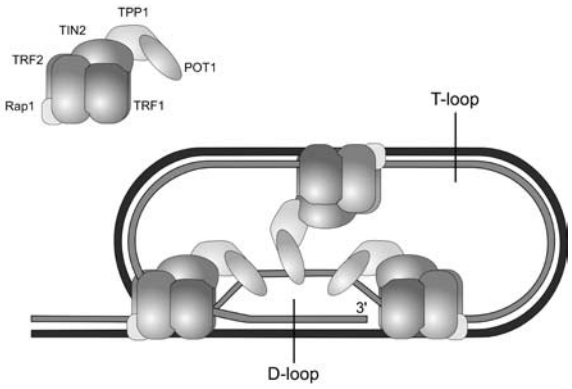

Figure 2. Schematic representation of the telomere shelterin complex and its associated proteins; TRF1, TRF2, POT1, Rap1, TIN2, and TPP1.

relatively easily obtained and processing is a relatively simple process. Telomere length in leukocytes is highly variable among individuals and decreases throughout life. Especially large differences develop the first few years after birth[20] after which telomere length are relatively stable throughout childhood, preadolescent, and adolescent years. Eventually, telomere length attrition increases at very old age (Figure 3). An important aging hypothesis is that telomere attrition increases at the onset of disease. Therefore, telomere length of the leukocytes could be a good marker for disease. Telomere length in different aging diseases is discussed later in the review. The major disadvantage of using leukocyte telomere length is that it is a measure of the activity state of the immune system and one might argue that leukocyte telomere length is rather a representation of increased inflammation than of aging.

Most animal research on telomeres has been performed in rodents, especially on inbred mice and rat species that have highly variable telomere lengths. Laboratory rats have relatively long telomeres that vary between 20 and 100 kb and telomere length in mice is even more variable and can extend up to 150 kb (of the C57BL/6 mice).[21, 22] In contrast, the outbred (wild type) mice strain Mus spretus has telomere length that is comparable to human cells. Comparing multiple mice strains showed that most mouse species do not have long telomeres, and long telomeres in mice strains originate from excessive breeding.[21] In rats, telomere length shortens with aging in several organs like kidney, liver, pancreas, and lungs. Research into telomere length from blood derived cells from multiple bird species with different life expectancies shows that telomere attrition rate is a better predictor of life expectancy than the age of the animal.[23] One remarkable animal is the Leach's storm-petrel, a long lived bird species with a maximum observed lifespan of 36 years. Instead of telomere attrition, it is suggested that the telomere length increases during aging in this species.[23] The Leach's storm-petrel has increased levels of telomerase activity in their bone marrow cells compared to other birds.[24] It is tempting to speculate that these animals have managed

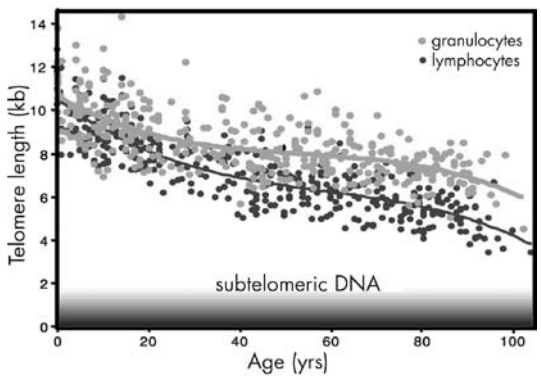

Figure 3. Telomere length in lymphocytes and granulocytes during human lifespan. Reproduced with permission from[127].

to increase their lifespan by dealing with telomere erosion. However, the assumption that absolute telomere length has an effect on life span is still elusive. For example, mice strains with longer telomeres do not seem to have an increased lifespan compared to mice strains with shorter telomeres. How these differences in telomere length affect lifespan are still unknown, the most accepted hypothesis is that the shortest telomeres are contributing most to the expected lifespan.[25]

Telomere biology and cellular senescence

Primary cells in culture are not immortal. As Hayflick demonstrated in 1961, cells stop dividing after a certain number of passages and become sedative[2], a phenotype also known as replicative senescence. The senescent phenotype is accompanied by changes in morphology, gene expression, and proteins. Beta galactosidase staining is frequently used to identify senescent cells and is associated with changes in p53, p16, and p21 expression.[26-28] There are multiple stimuli that can induce senescence; telomere shortening, DNA damage, and induction of oncogenic or tumor suppressor signals.[29-31] Induction of cellular senescence is an important suppressor of tumorigenesis.[30, 32] Although telomere attrition might not be primarily involved in the acute induction of senescence[29], the cumulative burden of oxidative stress and the cumulative telomere attrition might increase to likelihood of a cell to enter senescence.[31] Telomere attrition through replication and accumulation of DNA damage can result in an increase of senescent cells in different tissues and organs eventually resulting in decreased function and pathology. Telomere shortening has been implicated as one of the major mechanisms of replicative senescence.[14] The end-replication problem accounts for a loss of ~100 bp telomere length at each population doubling. On

average cells are estimated to reach senescence after ~50 population doublings. This is a bit earlier than predicted by the end-replication problem alone. It is likely that the state of the telomere and the presence of the proteins involved in forming the shelterin complex are important cofactors associated with the induction of senescence.[33] For example, there is ample evidence that disruption of the telomere binding proteins results in early senescence. In primary human fibroblasts, TRF2 inhibition induces a p53- and retinoblastoma-dependent senescent phenotype.[33, 34] Likewise, inhibiting POT1 by RNA interference led to the disappearance of the telomeres single-stranded overhangs and induced apoptosis, chromosomal instability, and senescence.[35]

Telomere biology in stem cells

Stem cells and progenitor cells have an important role in maintaining tissue homeostasis by replenishing (senescent, apoptotic) cells and repairing damage that occurs throughout life. Exhaustion of the stem cell or progenitor cell pool has been considered an important factor in the aging process of an organism.[36] One of the hallmarks of stem cells is their telomerase activity and stable length of their telomeres.[37, 38] Stem cells reside in different compartments throughout the body. In mice it has been shown that there exists a large difference in telomere length among the different compartments. The longest telomeres have been observed in skin, small intestine, cornea, testis, and brain compartments.[39] Although it seems that stem cells have stable telomeres by its increased telomerase activity, it does not make them invulnerable to telomere erosion. Clonal expansion after damage or in a disease state could induce telomere erosion that ultimately could induce senescence and an exhaustion of the stem cell pool. This hypothesis is supported by data from bone marrow-derived cells exhibiting a decreased migratory capacity and significant telomere shortening in patients with coronary artery disease.[40] The best characterized stem cells are the hematopoietic stem cells (HSC) which continuously replenish the hematopoietic cell lineages. HSC have been reported to have shorter telomeres compared to fetal liver and cord blood derived cells.[41] Recent data have also suggested a reduction of telomere length of HSC during aging.[42]

Telomere biology and premature aging

Some human disorders associated with shorter telomere length originate from defective telomerase function or mutations in the DNA repair system. For example, Dyskeratosis congenita (DC) is a human premature aging syndrome linked to mutations in the telomerase complex resulting in decreased telomerase stability and shorter telomeres.[43] Patients with

DC develop numerous different pathologies, including short stature, hypogonadism, infertility, bone marrow failure, skin defects, hematopoietic defects, and premature death. In addition, these patients have an increased susceptibility to develop malignancies. Another human disease example that involves a telomerase mutation is aplastic anemia. Subjects with aplastic anemia experience accelerated telomere shortening and die young.[44] Some diseases originating from mutations in genes of the DNA repair system also result in a phenotype characterized by accelerated telomere shortening and premature aging. Example genes include the Ataxia telangiectasia (ATM), Werner syndrome, Bloom syndrome, and Fanconi anemia genes. Most of these DNA repair genes also have a role in telomere biology. Mouse knockout models for these proteins do not always result in the same characteristics as the human disease. It has been suggested that the remarkable longer telomere length in mice might provide an explanation for these discrepancies. Combining DNA repair KO mice for Werner, Bloom, and ATM syndrome with the TERC$^{-/-}$ mice indeed resulted into a phenotype with characteristics that more closely resembled the expected pathology in humans.[45]

Telomeres and aging associated diseases

The debate on how telomere biology affects life span is ongoing, but a link between telomere length and mortality has been established. In addition, numerous associations between aging-associated diseases and telomere length have been reported.[46] Telomere length could be considered as a biological parameter that intertwines replicative history and exposure to environmental stress. Human life span is highly dependent on the development of aging associated diseases especially cancer and cardiovascular disease.

Cancer

Tumorigenesis is a major factor influencing life expectancy in long-lived species. Shorter telomere length is also a risk factor for the development of cancer.[47] Progressive shortening of the telomeres will lead to activation of the DNA damage response.[48, 47] In the normal situation this will result in activation of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia- and Rad3-related (ATR), and the associated downstream factors including CHK1, CHK2, and phosphorylation of p53. In the setting of a competent p53 pathway, senescence or apoptosis will be initiated and tumorigenesis inhibited.[49] However, when the p53 pathway is inadequate tumorigenesis is no longer inhibited in the presence of telomere dysfunction.[48-50] In addition, 80 to 90% of all tumors express telomerase or have a form of alternative telomere lengthening.[51] Clinical data revealed that telomere length (measured in lymphocytes) is shorter in subjects with different types of cancer, including cancers of the head and neck, breast, bladder, prostate, lung, and kidney.[47]

Cardiovascular risk factors

Next to cancer, cardiovascular disease and its risk factors are the major contributors to the population's disease burden during aging. For example, the presence of diabetes has been linked to reduced telomere length.[52-54] In the Framingham Heart Study, even a subclinical presence of insulin resistance was associated with reduced telomere length.[55] Hypertension and the responsiveness to angiotensin are related to outcome in humans.[56, 57] The number of genetic variants influencing the development of hypertension is only small.[58] However, a role for telomeres has been suggested, normotensive persons with short telomeres were more susceptible to develop hypertension and hypertensive subjects with short telomeres were more susceptible to develop atherosclerosis.[59] Interestingly, even subclinical activation of the renin-angiotensin system (RAS) has been associated with shorter telomeres.[55, 60] A final example is cigarette smoking, a strong risk factor for the development of coronary heart disease.[61] Smoking negatively affects telomere length[62-64], possibly due to mechanisms involving oxidative stress.[65]

Atherosclerosis

Endothelial dysfunction is recognized as one of the earliest events of atherogenesis[66] and is associated with classical risk factors or risk markers including cholesterol and inflammatory markers[67, 68], which can be modified by pharmacological treatment.[57] The endothelial and smooth muscle cells in the vessel, which are most susceptible to develop atherosclerosis are highly proliferative and are subjected to stress by increasing mean arterial pressure, increased cholesterol, and increased oxidative stress. This results in an increased susceptibility for senescence.[69, 70] Indeed senescent-positive endothelial cells can be found in almost any atherosclerotic plaque[71] and an association with shorter telomeres in atherosclerotic plaques has been established.[72] The first clinical study linking coronary artery disease to telomere length dates back to 2001.[73] This ground breaking study suggested telomeres of circulating white blood cells to be approximately 300 bp shorter in patients with coronary artery disease compared to controls. The authors estimated that this telomere differences resembles an age difference of almost 9 years.[73] Further and larger scale studies confirmed these findings and extended it to premature atherosclerosis and ischemic heart failure.[46, 74-76] For example, the West of Scotland Primary Prevention Study (WOSCOPS) observed that subjects in the middle or lower tertile of telomere length were at greater risk to experience a clinical manifestation of coronary heart disease than persons with longer telomeres.[75] The WOSCOPS data also suggested that the use of statins was more beneficial for the patients with the shortest telomeres.[75] Apparently patients that are protected by longer telomeres addition of statin treatment did not result in additional protection. Interestingly, telomeres of offspring from subjects with coronary artery disease already have shorter telomeres compared to offspring from

parents without atherosclerosis.[77] This might explain part of the heritability of coronary artery disease next to other genetic factors.[78, 79]

Heart failure

Chronic heart failure (CHF) is the main cardiovascular discharge diagnosis in the United States.[80] In particular, after the necessity of hospital admission, CHF is associated with a high mortality rate.[81] Recent clinical trials have not added much to the prognosis, and the search for new strategies is intensive.[82-85] Although in general, the incidence and prevalence of CHF steeply increases with aging, there exists a striking variability in the susceptibility, age of onset and pace of progression. This variability cannot completely be attributed to the presence of conventional risk factors and recent evidence is suggesting a role for telomere biology.[86] Endomyocardial biopsies from patients with heart failure have demonstrated that diseased hearts are characterized by shorter telomeres, increased cellular senescence, and cell death.[87] It has been estimated that telomere length is reduced by as much as 25% in failing hearts compared to nonfailing hearts.[88] Also, the telomere length in leukocytes of subjects with heart failure are significantly shorter compared to age and gender balanced controls.[89] In this study, the severity of heart failure symptoms was also associated with the degree of telomere shortening. Furthermore, cardiac function as measured by ejection fraction in general has been associated with telomere length.[90] One standard deviation of longer telomere length was associated with a 5% higher left ventricular ejection fraction. In these elderly subjects, telomere length alone accounted for 12% in the observed variability of ejection fraction. Renal function impairment relates to even worse outcome in patients with CHF. Shorter telomere length in CHF is also associated with decreased renal function, possibly due to drop-out of functional nephrons.[76]

Telomere maintenance

Telomerase, a ribonucleoprotein complex that is composed of RNA and protein components, can elongate the telomere sequence in mammals and yeast by binding to the open end of the G-strand. Telomerase is highly expressed during embryonic development but its expression is suppressed within a few weeks after birth in most somatic cells. Highly proliferative cells maintain high levels of telomerase, like stem cells, progenitor cells, lymphocytes, skin keratinocytes, and cancer cells.[91] The major components of the active telomerase complex are telomerase reverse transcriptase (TERT), a telomerase RNA component (TERC, that is complementary to the telomere sequence) and dyskerin, which is a protein that binds to both TERT and TERC and increases stability of the complex.[92, 93] Elongation of the telomeres in mammals and yeast not depending on telomerase is called

alternative lengthening of telomeres (ALT). In human tumors, it was discovered that cells negative for telomerase could also elongate their telomeres by a recombination mechanism.[94] Recombination takes place by binding of the ALT-associated promyelocytic leukemia bodies to the telomeres. Telomere elongation occurs heterozygous in these cells and ALT can best be recognized by the presence of both short and long telomeres.

The TERC$^{-/-}$ mouse has increased our knowledge on the importance of telomerase in aging and in the potential role of telomerase and telomere shortening in the different diseases. As always, it is difficult to translate data from knockout models directly to human pathology, but especially in premature aging diseases the TERC$^{-/-}$ mice show great overlap with human disease.[45]

The telomerase-deficient mice

Telomerase-deficient mouse models

The notion that mouse and human tumors express elevated levels of telomerase activity, while normal adjacent tissue lacks measurable activity, led to the development of models to study telomere biology. In 1997, Dr Maria A. Blasco reported the successful generation of telomerase-deficient mice by knocking out the RNA template of telomerase (TERC$^{-/-}$ mice).[8] This mouse model was established to serve as a tool to study tumor formation and cell viability in the absence of telomerase. Besides the RNA template, another essential component of telomerase is the TERT. Not surprisingly, TERT$^{-/-}$ mice have also been constructed.

Although originally generated to serve research related to oncology, the telomerase-deficient mice have provided valuable information for age-associated diseases, including those related to cardiovascular disease.

General phenotype of the telomerase-deficient mouse

The telomeres of the TERC$^{-/-}$ mice shorten at a rate of ~5 kb in every subsequent generation.[8] Therefore, experiments using TERC$^{-/-}$ mice commonly involve several generations (G) of these mouse compared with wild types. Telomere length in G3 TERC$^{-/-}$ mice decreases as much as 50% during ageing from 1 to 16 months.[95] TERC$^{-/-}$ mice of later generations show increasingly severe structural abnormalities on cellular level, including undetectable short telomeres, aneuploidy, and chromosomal end-to-end fusions.[8] In general, animals with phenotypical features of ageing have shorter telomeres than age-matched controls from the same generation lacking a clear ageing phenotype. Later generations TERC$^{-/-}$ mice show a decreasing life span (Table 2). Autopsy of late generation spontaneously diseased animals could not identify a clear cause of death, comparable to natural death in humans.[95] Later

Table 2. Characteristics of generations of TERC$^{-/-}$ mice.

	WT	G1	G2	G3	G4	G5	G6
Lifespan	Normal	Normal	Normal/reduced[a]	Normal/reduced[a]	Normal/reduced[a]	Normal	Reduced
Aging phenotype (hair graying, alopecia)	Normal	—	—	Accelerated	—	—	Accelerated
Body weight	Normal	Normal	Normal	Normal/20% reduction[a,b]	20% reduction[a,b]	15% reduction	20–25% reduction[c]
Blood pressure	Normal	Hypertensive[a]	—	Hypertensive[a]	—	—	—
Left ventricular function	Normal	—	Slightly impaired	—	—	Severely impaired	—
Myocyte size	Normal	—	Increased	—	—	Severely increased	—
Angiogenic potential	Normal	—	Normal	Decreased	Decreased[a]	Decreased[a]	Decreased
Wound healing	Normal	Normal	—	Impaired	—	—	Impaired

[a]Observed in mice with C56Bl/6 genetic background, all other observations were in mice with originally mixed background.
[b]Observed in animals >4 months of age.
[c]Observed in animals >6 months of age.

generation and aged TERC$^{-/-}$ mice show many more defects than earlier generation and younger TERC$^{-/-}$ mice. These defects include reduced body size and weight, hair graying and loss, infertility and testicular atrophy, spleen atrophy, signs of immunosenescence, and bone marrow proliferative defects.[95, 96] Wound healing is also impaired in aged animals TERC$^{-/-}$.[95] Reduced angiogenic potential of these mice has been suggested as an explanation for this observation. Below we will focus in more detail on the knowledge of the angiogenic potential in these mice.

Although some interesting studies have been performed on TERT$^{+/-}$ mice, these mice are substantially less well-studied compared with TERC$^{-/-}$ mice. The construction of the TERT$^{+/-}$ mice has first been reported in 1999.[97] Both the TERT$^{+/-}$ and the TERC$^{-/-}$ mice do not show significant phenotypic abnormalities at early age in the first generation. Unfortunately, information on the general phenotype of the TERT$^{+/-}$ mice of later generations is sparse and data on fertility of these mice are reported differently by different groups, possibly due to different backgrounds of the mice. Unchanged litter size in G1 and G2 TERT$^{+/-}$ mice (progeny from a chimeric and a wild-type C57Bl/6 mouse) has been reported by some[97], while others experienced dramatically reduced litter size in G2 TERT$^{+/-}$ mice on a pure C57Bl/6 background.[98] Indisputable is the fact that later generation TERT$^{+/-}$ and TERC$^{-/-}$ mice both have considerably shorter telomeres than their wild-type litter mates.[98-100]

In general, homozygous TERT and TERC deficient mice display a similar phenotype. However, one remarkable difference between heterozygous TERT and TERC deficient mice has been observed. In contrast to TERC$^{+/-}$ mice, TERT$^{+/-}$ mice do not have detectable defects in telomere maintenance and elongation. In both heterozygous mice, the mRNA levels of their knocked out gene are approximately 30–50% of that of wild-type mice. The differences are therefore unlikely to be attributable to differences in target gene expression. Presumably, both TERC and TERT are essential for telomere maintenance and elongation, but in contrast to TERT, gene copy number and transcriptional regulation of TERC are limiting for telomerase activity.[100, 101]

Hypertension

Several cross-sectional human studies associate blood pressure parameters with telomere length of circulating leukocytes.[55, 102] Only one study is available evaluating blood pressure in telomerase-deficient mice.[103] In conscious mice, tail sphygmomanometry revealed that TERC$^{-/-}$ mice from G1 showed higher systolic blood pressures compared with wild type. In G3 mice, both systolic and diastolic blood pressures were increased compared with wild-type and G1 mice. Studying anaesthesized G3 and wild-type mice with invasive haemodynamic studies did not show functional modifications in the nitric oxide system or the responsiveness to angiotensin II. Thus, the differences in blood pressure do not seem to be attributable to these two systems. Interestingly, the response to endothelin—a powerful

vasoconstrictor—was diminished in G3. In concordance, treatment with bosentan, an endothelin receptor antagonist, resulted in a more pronounced drop of blood pressure in G3 mice compared with wild-type. Furthermore, plasma and urine endothelin levels were gradually and significantly increased in G1 and G3 mice. To further explore the role of endothelin in the observed differences in blood pressure between $TERC^{-/-}$ and wild-type mice, mRNA expression of endothelin converting enzyme (ECE) was measured. $TERC^{-/-}$ mice showed higher mRNA expression of ECE-1 and specific inhibition of ECE only reduced blood pressure in $TERC^{-/-}$ mice. *In vitro* experiments with transfection of deletion mutants of the ECE-1 promoter suggested that the AP-1 binding sequence of the ECE-1 promoter is involved, so that transcriptional control of ECE-1 may be disrupted in $TERC^{-/-}$ mice.[103] Whether long-term treatment with endothelin receptor blockers is beneficial in $TERC^{-/-}$ mice remains to be determined. In addition, this functional data obtained in the $TERC^{-/-}$ mice have not been translated back to human studies, meaning we do not know yet whether ECE activity is also involved in the association between hypertension and telomere length in humans.

Atherosclerosis

The association between reduced telomere length in leukocytes with the development and presence of atherosclerotic manifestations in humans is demonstrated by several independent groups.[72, 73, 104, 105] As mice in general are resistant to the development of atherosclerosis,[106] many groups are using the apolipoprotein-E (ApoE)-deficient mice, which do develop atherosclerosis when exposed to a high fat diet.[107] Interestingly, $ApoE^{-/-}$ mice were inter-crossed with $TERC^{-/-}$ mice to study the effects of telomeres on the development of atherosclerosis.[108] Fourth generations of $TERC^{-/-}ApoE^{-/-}$ and $TERC^{+/+}ApoE^{-/-}$ mice were challenged with a high cholesterol high fat diet. Remarkably, generation four (G4) $TERC^{-/-}ApoE^{-/-}$ mice developed less atherosclerotic lesions compared with G4 $TERC^{+/+}ApoE^{-/-}$ mice (Table 3). In addition, the atherosclerotic plaques of G4 $TERC^{-/-}ApoE^{-/-}$ mice were morphologically in a less advanced stage of atherosclerosis, compared with G4 $TERC^{+/+}ApoE^{-/-}$. This suggests that the absence of telomerase activity is protective for atherosclerotic disease. These observations could not be attributed to differences in serum cholesterol levels.[108] It was also observed that the proliferative capacity of macrophages and lymphocytes was decreased in G4 $TERC^{-/-}ApoE^{-/-}$ mice compared with G4 $TERC^{+/+}ApoE^{-/-}$, suggesting reduced inflammatory capacity. This might explain the differences in atherosclerotic manifestations in this experimental model, since progression of atherosclerosis is partly dependent of functional immunocompetent cells.[108]

Angiogenesis

Evidence for involvement of telomerase in angiogenesis has been provided by adenovirus-mediated transfer of TERT in the rat hind limb ischaemia, which enhances capillary density

Table 3. Mouse models of telomerase manipulation and the cardiovascular phenotype.

Model	Animals (genetic background)	Cardiovascular phenotype	Ref. no.
Telomerase deficiency	Mouse, TERC$^{-/-}$ (C57Bl/6 background)	Hypertension	103
		Decreased angiogenic potential (>generation 3)	110
		Inflammation: decreased Lymphocyte (T and B) proliferative response	116
	Mouse, TERC$^{-/-}$ (mixed background)	Myocyte hypertrophy, left ventricular failure and dilatation	111
		Decreased angiogenic potential (>generation 3)	110
	Mouse, TERT$^{-/-}$ (mixed background)	Inhibited upregulation of telomere-stabilizing protein TRF2 following exercise. No protection against doxorubicin-induced cardiomyopathy	112
	Mouse, TERC$^{-/-}$ ApoE$^{-/-}$	Less atherosclerosion, decreased proliferative capacity of inmunocompetent cells	108
	Mouse, TERC$^{-/-}$ Atm$^{-/-}$	Depletion of haematopoietic progenitor cells	123
	Mouse, TERC$^{-/-}$ Wrn $^{-/-}$	Development of diabetes mellitus type 2	124
TERT depletion	Rat, transfection of double negative TERT	Abrogation of angiogenesis	109
TERT overexpression	Mouse, transfection of cardiac-specific TERT	Myocyte hypertrophy, left ventricle hypertrophy without diminished function, increased cardiac ischemia tolerance in myocardial infarction	125

in the ischaemic tissue.[109] Also, TERC$^{-/-}$ mice have been studied using both matrigel implants and murine melanoma grafts.[110] In an *in vivo* matrigel assay, late generation TERC$^{-/-}$ mice showed diminished angiogenic potential compared with wild-type mice. Early generation TERC$^{-/-}$ mice, who have normal telomere length, did not have impairment of angiogenesis as assessed by the *in vivo* Matrigel assay.[110] This suggests that short telomere length limits the angiogenic potential, and not the absence of functional telomerase itself. Also an *in vivo* angiogenesis model using murine melanoma cells showed decreased tumor formation efficiency and growth rate in later generations TERC$^{-/-}$ mice. Microvessel density in tumor cryosections was stained with an anti-CD31 antibody—an endothelial cell marker—and it was shown that the microvessel density of G5 TERC$^{-/-}$ tumors was only half of the wild-type and G2 TERC$^{-/-}$ tumors.[110]

Cardiac myocytes and ventricular failure

The effects of telomerase deficiency on cardiac myocyte size, number, proliferative potential, and myocyte apoptosis has been studied in combination with cardiac function in G2 and G5 TERC$^{-/-}$ mice of the original mixed background and compared with wild-type mice. The progressive decrease of telomere length in cardiomyocytes of successive generations of TERC$^{-/-}$ mice was associated with an increase of p53 expression.[111] G5 TERC$^{-/-}$ mice suffer from severe left ventricular failure, characterized by increased end diastolic left ventricular pressure, decreased maximally developed left ventricular pressure and disturbed contractility and relaxation of the left ventricle. These mice also showed anatomical changes of the heart, similar to dilated cardiomyopathy in human, together with decreased total number of myocytes and increase of myocyte hypertrophy. In addition, apoptosis of myocytes was an approximate 40% greater in G5 TERC$^{-/-}$ mice, compared with wild-type and G2 TERC$^{-/-}$ mice.[111] In G2 TERC$^{-/-}$ mice, only a slight decrease in left ventricular pressure compared with wild-type mice was observed.[111] These data suggest that late generation of TERC$^{-/-}$ mice spontaneously develop pathological cardiac remodelling and severe ventricular dysfunction. Another study showed that exercise increased TRF2 expression and prevented doxorubicin-induced cardiac apoptosis in wild-type mice, but not in TERT$^{-/-}$ mice. This suggests that, in absence of telomerase, upregulation of telomere-stabilizing proteins is challenged and cardiac apoptosis is more severe.[112]

In conclusion, telomerase-deficient mice provide a model to study the efficacy of telomerase-based therapies for heart failure. However, it should be taken into account that the majority of patients who develop heart failure have coronary artery disease.

Stem cell biology and tissue regeneration

A key process in tissue and organ homeostasis is the mobilization of stem cells for maintenance and repair. Evidence is supporting a role for bone marrow derived cells in the maintenance and regeneration the endothelium.[113] More controversial is whether the heart is

also harbouring progenitor cells in adult life.[114, 115] The TERC$^{-/-}$ mice have provided us with more insights in the role of telomerase and telomere length in several well-characterized stem-cell subtypes, including haematopoietic, epidermal, and neural stem cells.

Haematopoietic progenitor cells from G1 TERC$^{-/-}$ mice have a normal capacity to grow and differentiate *in vitro*. Mature haematopoietic organ structure and function seem to be well compensated in TERC$^{-/-}$ deficient mice, as no changes in peripheral blood count and profile were observed through successive generations and mature immunocytes show normal responses to mitogenic or infectious stimuli.[116] However, *in vitro* haematopoietic colony-forming unit (CFU) assays revealed that later generation TERC$^{-/-}$ mice have a significant decrease in total number of CFU-granulocyte–monocyte, CFU-granulocyte, -erythrocyte, -monocyte, -megakaryocyte, and decreased high-proliferative-potential colony forming cell colonies.[116] In addition, serial and competitive transplantations of TERC$^{-/-}$ bone marrow stem cells showed reduced long- term repopulating capacity compared with wild-type cells.[117, 118] This indicates that long-term renewal of haematopoietic stem cells is compromised upon telomere loss.

In different generations of TERC$^{-/-}$ inbred mouse, the epidermal stem cell number has been compared.[119] In G1 and even more pronounced in G3 TERC$^{-/-}$ mice, greater numbers of epidermal stem cells were present in the bulge area of the hair follicle. Interestingly, the epidermal stem cells in TERC$^{-/-}$ mice showed a defect in their mobilization. Coincidentally, the proliferation index in different compartments was lower than that of wild-type follicles. In addition, *in vitro* culture of keratinocytes from G1 and G3 TERC$^{-/-}$ mice formed fewer and smaller colonies than those of wild type.[119] Thus, epidermal stem cells in TERC$^{-/-}$ mice are less functional than in wild-type, and the increased numbers of epidermal stem cells in the TERC$^{-/-}$ mice are possibly due to accumulation in the follicles, as the epidermal stem cells have impaired capacity to mobilize.

Although bone marrow and stem cells are considered important in the pathogenesis and possible treatment of cardiovascular disease, including atherosclerosis and heart failure, studies focussed on the role of telomere biology are lacking. In this regard also, the TERC$^{-/-}$ model provides a good model to study the efficacy of stem cell-based therapies for heart failure.

Conclusions and future perspectives

Telomere biology is involved in biological aging and disease processes. Experimental evidence suggests that telomere shortening, uncapping, and cellular senescence results in an "aging" phenotype.[33] The exhaustion of progenitor cells and the cumulating of senescent cells might explain the decline in organ function associated with aging. Shorter telomere length has been associated with several age associated diseases, including cancer, diabetes, atherosclerosis, and heart failure. To gain more insights in the role of telomere biology in the aging process of humans, we are still in need of large population based cohorts with telomere length and telomerase activity measurements at multiple time-points. If telomere biology can be proven to be causally involved in the development and progression of these age-associated diseases, it will pave the way for new therapeutic or preventive strategies. For example, telomerase or telomere length could be targeted in the emerging stem cell therapies for organ dysfunctions.

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Chapter 3

Telomerase knockout mice have an impaired running capacity

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Manuscript in preparation.

Abstract

Objective

Physical activity increases life span and is thought to postpone biological aging and aging associated diseases. Telomerase is an essential enzyme in telomere maintenance and in its absence biological aging is accelerated. Here we investigated the effects of physical exercise on cardiovascular function in wild type (WT) and telomerase deficient mice ($TERC^{-/-}$).

Methods and results

C57BL/6 (WT), $TERC^{-/-}$ generation 2 (G2), and generation 3 (G3) mice were randomized to voluntary physical activity (unlimited access to a running wheel) or sedentary 'life-style'. The voluntary running wheel performance of $TERC^{-/-}$ mice was considerably lower compared to WT mice. Differences in left ventricular function did not explain the difference in physical activity. Physical activity was associated with a decreased resting heart rate in WT and $TERC^{-/-}$ mice. In WT but not in $TERC^{-/-}$ mice, physical activity improved endothelial function. Histological analysis of the gastrocnemius muscles showed a marked decrease of the high oxidative capacity fibers in sedentary $TERC^{-/-}$ G3 mice, but this was completely restored in the $TERC^{-/-}$ G3 running group.

Conclusions

Lack of telomerase expression is associated with decreased voluntary wheel-running performance in a murine exercise model. There were no gross abnormalities in cardiac function, but a combination of multiple small changes, including a difference oxidative capacity of the muscle fibers and maximal endothelial vasodilatation, might account for the decreased exercise activity.

Introduction

Regular non extensive exercise is associated with an increased life span[1] and is thought to postpone the biological aging process and aging associated diseases. Voluntary regular exercise has been reported to reduce cardiovascular events.[2, 3] In diabetic patients regular physical activity also improves insulin sensitivity.[4, 5]

The mechanisms trough which physical activity exerts its beneficial effects are not completely elucidated. Paradoxically, more exercise increases physiological stress and increases the level of reactive oxygen species (ROS) leading to DNA damage.[6] On the other hand, exercise causes upregulation of the anti-oxidative stress related pathways and may be protective. It has been suggested that the effects of exercise might be U-shaped and that excessive exercise will not have beneficial effects or might even be harmful.[7]

Adults with higher levels of physical activity have on average longer telomeres than inactive age matched controls.[8, 9] However in a Chinese population after the age of 70, telomere length was not associated with exercise anymore.[10]

Recent studies show that exercise increases telomerase activity in cardiac and vascular tissue of mice and increases telomere-stabilizing proteins in leucocytes of athletes. Increase in telomerase is associated with decreased levels of apoptosis and senescence in the different organs.[11, 12] Shorter telomere length has been associated with cardiovascular diseases including heart failure[13], hypertension[14, 15] and atherosclerosis.[16, 15] Telomerase deficient mice ($TERC^{-/-}$) show an accelerated aging phenotype that is accompanied by hypertension and decreased angiogenic potential.[17] We investigated whether physical exercise can protect telomerase deficient mice from accelerated deterioration in cardiovascular function.

Methods

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Male and female C57Bl/6j WT mice and C57BL/6 mice lacking the RNA component of the enzyme telomerase ($TERC^{-/-}$)[18] generation 2 (G2) and $TERC^{-/-}$ generation 3 (G3) were obtained from Centro Nacional de Investigagiones Oncológicas (CNIO) Spain. During the entire experiment, animals were kept on a 12 hour light, 12 hour dark cycle with *ad libitum* access to food and water.

Voluntary exercise

At the age of 12 weeks, 4 animals (2 male, 2 female) from WT, G2 and G3 groups were

subjected to 4 weeks of voluntary exercise. The animals were individually housed and the cages were equipped with a running wheel and a tachometer. Daily notations of the time spent in the wheel, the distance travelled and the average speed were taken. Sedentary animals were single caged for the same time period as exercise animals.

Echocardiographic and hemodynamic measurements

Cardiac dimensions were measured when the animals were subjected to voluntary wheel running or animals were individually caged as sedentary controls. Cardiac dimensions were measured using transthoracic echocardiography with a 14 MHz transducer (Vivid 7, GE Healthcare, Diegem, Belgium), mice were anesthetized using isoflurane (2% in O_2), temperature was maintained by placing the mouse on a heating pad. Short-axis view and M-mode tracings were used to determine end-diastolic and systolic left ventricle (LV) interventricular septal thickness (IVS). Mitral valve Doppler signals were used to establish E/A ratio. Relative wall thickening was calculated by the formulas: wall thickness changes, ΔWT = ESWT (end systolic wall thickness) − EDWT (end systolic wall thickness), and relative wall thickness, %WT = 100% * (ΔWT/EDWT).

Heart rate (HR) and mean arterial pressure (MAP) were measured at sacrifice using a Millar catheter (Mikro-tip 1.4F; SPR-839, Millar Instruments, Houston, TX, USA). Mice were anesthetized as described above and the right carotid artery was inserted with the pressure transducer catheter. After the hemodynamic measurements, mice were sacrificed by excision of the heart.

Vascular function

Thoracic aorta was collected after sacrifice, carefully cleaned and thereafter mounted on a small wire myograph (Danish Myo Technology A/S, Aarhus, Denmark). After normalization, vessels were pre-contracted with Phenylephrine (PE) (Sigma-Aldrich, Zwijndrecht, The Netherlands) and vasodilatation was measured by dilating the vessel with increasing concentrations of acetylcholin (Ach) (Sigma-Aldrich), endothelium-independent relaxation was measured by 0.1 mmol/L sodium nitroprusside (Sigma-Aldrich).

Histology

Sections for histological staining were cut at 10 μm thick from frozen gastrocnemius muscle and were place on polysine coated microscope slides. NADH tetrazolium reductase (TR) staining was carried out by incubating slides for 45 minutes with NADH-TR reaction solution (0.2 mol/LTris, 1.5 mmol/L NADH and 1.5 mmol/L nitrotetrazolium blue) followed by 4% formaldehyde fixation and mounting the slides with Kaiser's glycerol gelatine (Merck KGaA. Darmstadt, Germany) Fibers that stained moderately or strongly for NADH-TR were counted positive for NADH-TR, from each animal at least 70 fibers were counted.

Telomere length measurement by PCR

Telomere length was determined in genomic DNA isolated from the spleen, by Nucleospin tissue kit (MACHEREY-NAGEL, Düren, Germany). The spleen was used, as this organ contains a high amount of lymphocytes, which would best represent blood measurements. Telomere length was determined by adapting the previous described protocol.[13] In short, relative telomere length was determined with ABsolute QPCR SYBR Green ROX Mix (Abgene, Epsom, United Kingdom) in the presence of 50 ng genomic DNA and 200 nmol/L forward and reverse primers. Real-Time PCR was conducted on the Biorad CFX384 (Biorad, Veenendaal, The Netherlands). Telomere (TEL) quantity was normalized by correcting for the single copy gene quantity of hemoglobin (HBB). Primer sequences can be found in Table 1.

RNA analysis

Total RNA from tissues was extracted with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). cDNA synthesis was performed using standard methods. Gene expression was measured with ABsolute QPCR SYBR Green ROX Mix (Abgene) in the presence of 10 ng cDNA and 200 nmol/L forward and reverse primers. Real-Time PCR was conducted on the Biorad CFX384 (Biorad). ANP levels were calculated by the CFX manager version 1.1 (Biorad) software, by the delta delta Ct method. GAPDH and β-actin levels were used as housekeeping genes. Primer sequences can be found in Table 1.

Table 1. Primer sequences used in Real-Time PCR

Primer	Forward	Reverse
TEL	5′-CGGTTTGTTTGGGTTTGGGTT TGGGTTTGGGTTTGGGTT-3′	5′-GGCTTGCCTTACCCTTACCCT TACCCTTACCCTTACCCT-3′
HBB	5′-ACAGCTCCTGGGCAATATG-3′	5′-GGGCTTAGTGGTACTTGTG-3′
ANP	5′-ATGGGCTCCTTCTCCATCAC-3′	5′-TCTACCGGCATCTTCTCCTC-3′
36B4	5′-GCTTCATTGTGGGAGCAGACA-3′	5′-CATGGTGTTCTTGCCCATCAG-3′
GAPDH	5′-CATCAAGAAGGTGGTGAAGC-3′	5′-ACCACCCTGTTGCTGTAG-3′
β–actin	5′-CGAGCGTGGCTACAGCTTCA-3′	5′-AGGAAGAGGATGCGGCAGTG-3′

Abbreviations: TEL: telomere, HBB: hemoglobin, ANP: atrial natriuretic peptide, 36B4: acidic ribosomal phosphoprotein PO, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

Results are expressed as mean ±SE. Groups were compared by unpaired Student's t-test or one-way ANOVA with Dunett post-hoc correction, non-Gaussian distributed data was compared by Kruskal-Wallis H test, followed by individual comparisons of means (Mann-Whitney U). Values of $P<0.05$ were considered statistically significant.

Results

TERC$^{-/-}$ mice have shorter telomere length

Telomere length was measured in the spleen of the different animal groups to establish relative telomere length differences between WT and TERC$^{-/-}$ G2 and G3 animals. As shown, in figure 1, relative telomere length in TERC$^{-/-}$ G2 mice was on average 2.2 fold lower than in WT mice. In TERC$^{-/-}$ G3 mice telomere length had further shortened and was on average 1.8 fold lower as compared to TERC$^{-/-}$ G2 mice (Figure 1).

TERC$^{-/-}$ mice have an impaired running performance

WT and TERC$^{-/-}$ mice were solitary housed and half of the cages were equipped with a running wheel, allowing voluntary exercise to half of the animals. All mice that had access to a running wheel also made use of it. In the first week of exercise, there was no difference in distance, time spent in the running wheel and average running speed between the different groups (Figure 2A-C). However, after the first week, differences between the groups became visible. Whereas the WT mice significantly increased the running distance in the second week and maintained this running distance in the weeks thereafter, the TERC$^{-/-}$ G2 and G3 mice

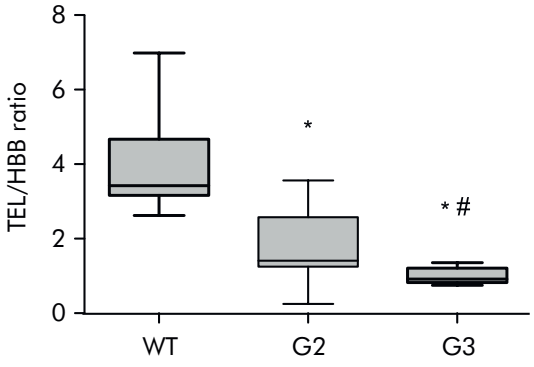

Figure 1. Telomere length in WT (n=7) and TERC$^{-/-}$ G2 (n=7) and TERC$^{-/-}$ G3 (n=7) mice. Relative telomere length measured by Real-Time PCR in genomic DNA isolated from the spleen. Data are depicted as a boxplot with median and interquartile range, whiskers represent minimum to maximum. * = $P<0.05$ compared to WT group, # = $P<0.05$ compared to G2 group.

did not increase their running distance (Figure 2A). After week one, the running wheel time became significantly different between WT mice and TERC$^{-/-}$ mice, this was mainly due to a decline in running time by the TERC$^{-/-}$ mice (Figure 2B). Based on the running wheel time and the total running distance we were able to calculate the average running speed during the different weeks. This revealed that all animal groups increased the average speed in their 4 weeks running time. This increase was, however, significantly higher in the wild type mice as compared to the TERC$^{-/-}$ mice (Figure 2C). Although not significant, we like to note that the TERC$^{-/-}$ G3 group performed less than the G2 group animals in all these parameters at all time points. Together these data indicate that TERC$^{-/-}$ G2 and G3 animals have a decrease in running wheel performance compared to WT animals.

TERC$^{-/-}$ and WT mice have similar cardiac function after 4 weeks of exercise

Next we investigated parameters that could potentially account for the differences in running wheel performance between WT and TERC$^{-/-}$ mice. Blood parameters were measured to determine whether signs of anemia or increased inflammation in TERC$^{-/-}$ mice were present (Table 2). Red and white blood cell counts were comparable between all three groups, and although hemoglobin and hematocrit values were somewhat elevated

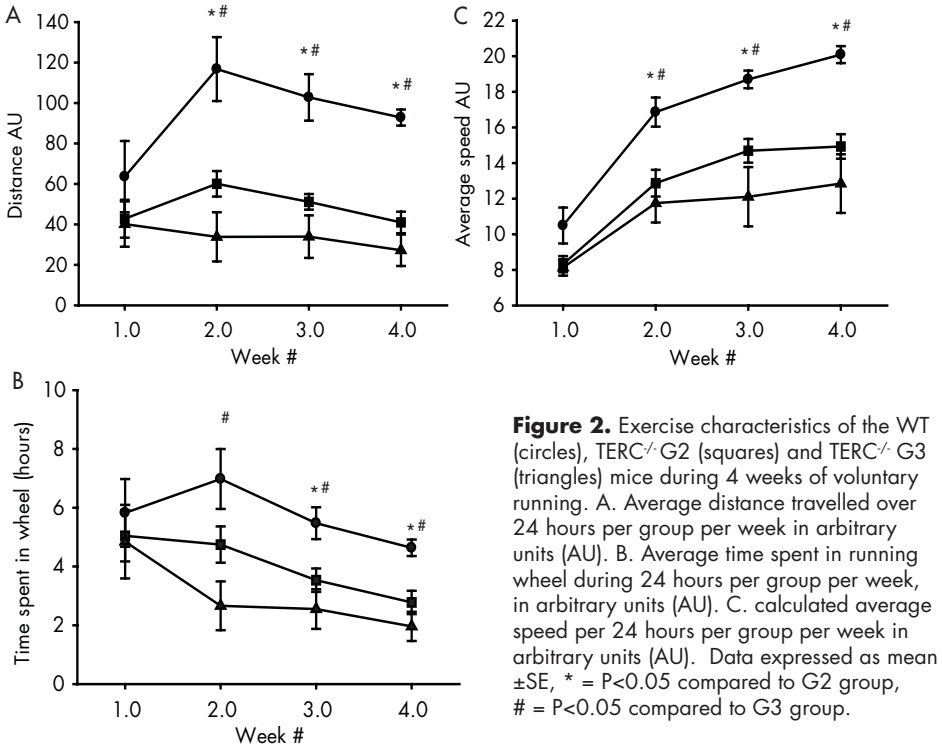

Figure 2. Exercise characteristics of the WT (circles), TERC$^{-/-}$ G2 (squares) and TERC$^{-/-}$ G3 (triangles) mice during 4 weeks of voluntary running. A. Average distance travelled over 24 hours per group per week in arbitrary units (AU). B. Average time spent in running wheel during 24 hours per group per week, in arbitrary units (AU). C. calculated average speed per 24 hours per group per week in arbitrary units (AU). Data expressed as mean ±SE, * = P<0.05 compared to G2 group, # = P<0.05 compared to G3 group.

Table 2. Blood characteristics of WT and TERC$^{-/-}$ sedentary and voluntary exercise mice

	WT nr (n=3)		WT vwr (n=4)		G2 nr (n=4)		G2 vwr (n=4)		G3 nr (n=3)		G3 vwr (n=4)	
WBC (10^9/L)	6.00	±1.91	5.45	±1.29	3.17	±0.62	5.20	±1.21	3.60	±1.00	4.30	±1.20
RBC (10^{12}/L)	8.89	±0.43	7.80	±0.60	9.46	±0.24	10.56	±0.48	7.21	±0.88	9.85	±1.18
HGB (mmol/L)	8.13	±0.43	7.23	±0.54	9.23	±0.09	10.55	±0.41*	7.03	±0.84	9.65	±1.16
HCT (L/L)	0.46	±0.03	0.39	±0.03	0.48	±0.01	0.54	±0.02*	0.37	±0.04	0.50	±0.06
PLT (10^9/L)	904.0	±105.7	826.5	±52.1	1114.7	±138.9	1107.0	±92.7	835.0	±242.6	1355.5	±124.6*

Data presented as means ±SE *P<0.05 versus WT vwr, one-way ANOVA with Dunett post-hoc correction.
Abbreviations: nr: non running. vwr: voluntary wheel running. WBC: white blood cell count. RBC: red blood cell count.
HGB: hemoglobin. HCT: hematocrit: PLT platelet count.

in the G2 running animals compared to the WT running animals and platelet count was elevated in G3 running animals compared to WT running animals there were no consistent differences.

To determine if TERC$^{-/-}$ mice had aberrant cardiac function before exercise, heart function was measured by echocardiography (Table 3). There was a difference in the percentage fractional shortening in TERC$^{-/-}$ mice compared to the WT animals. This was borderline not significant (P=0.054) for WT vs. G2, but significant for WT vs. G3 (P=0.044). These differences in fractional shortening did, however, not result in a changed ejection fraction or in a change in the cardiac output after correcting for body weight (Table 3).

Exercise can influence cardiac function resulting in physiological cardiac hypertrophy, which is characterized by an increased heart weight and an increase in cardiac atrial natriuretic peptide (ANP) expression levels.[19] After 4 weeks of voluntary exercise cardiac parameters were measured for all groups (Table 4). In line with previous studies with the TERC$^{-/-}$ mice, body weight decreased with each generation. Heart weight corrected for tibia length, a measurement of hypertrophy, did not show differences between the groups (Figure 3A). Also blood pressure and ANP levels measured in mRNA isolated from the left ventricle did not change between the groups (Table 4). These parameters indicate there was no hypertrophy in any of the groups.

Voluntary exercise resulted in all three groups in a 17% drop in the resting heart rate, which was measured at sacrifice at the end of the running period (Figure 3B), which was significant for the G2 group. Thus, although TERC$^{-/-}$ mice had a lower exercise performance,

Table 3. Cardiac characteristics at the beginning of the voluntary wheel running, measured by echocardiography

	WT (n=7)		G2 (n=8)		G3 (n=8)	
LV IVSs (mm)	1.20	±0.22	1.08	±0.49	1.00*	±0.50
LV IVSd (mm)	0.68	±0.26	0.61	±0.40	0.60	±0.20
relative wall thickening (%)	76.87	±8.89	78.91	±10.51	67.14	±8.02
ejection fraction (%)	73.57	±4.72	61.19	±3.21	60.26	±3.99
fractional shortening (%)	38.47	±4.25	28.36	±2.10	28.00	±2.60*
cardiac output (ml/min)	21.40	±1.48	15.50	±1.98*	16.55	±1.37
cardiac output corrected for body weight (ml/min/gr)	0.87	±0.05	0.68	±0.08	0.85	±0.09

Data presented as means ±SE *P<0.05 versus WT, one-way ANOVA with Dunett post-hoc correction. Abbreviations: LV: left ventricle, IVSs: interventricular septal thickness at systole, IVSd: interventricular septal thickness at diastole.

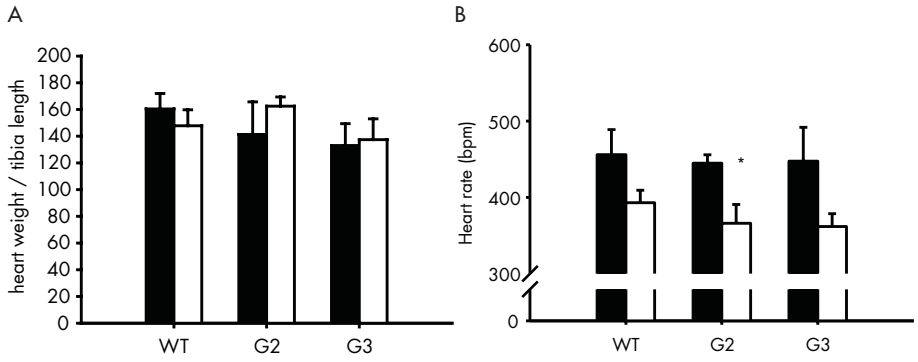

Figure 3. Cardiac parameters measured in sedentary and exercised WT and TERC$^{-/-}$ mice. A. Heart weight tibia length ratio after 4 weeks of exercise. B. Heart rate measured under anesthesia at the end of the end of the protocol. Data are expressed as mean ±SE, black bars represent non-running animals, white bars represent voluntary wheel running animals. * = P <0.05 compared to sedentary control of the same group.

a similar drop in resting heart rate was observed, suggesting that a similar physiological effect occurred in these mice.

Exercise improves maximum vascular dilatation in WT mice only

As exercise is potentially beneficial for endothelial function we measured endothelial function in the thoracic aorta by small wire myograph (Figure 4). Voluntary exercise improved acetylcholine induced maximal vasodilatation in PE pre-contracted vessels in the WT animals from 68.6 ±2.7% in the non-running group to 86.6 ±2.3% in the running group (P=0.004). In TERC$^{-/-}$ animals maximal relaxation of the sedentary groups was comparable to the sedentary WT group. No increase in maximal relaxation was, however, observed in the running TERC$^{-/-}$ groups. Thus, exercise improved endothelial function only in WT animals.

Sedentary TERC$^{-/-}$ G3 mice have lower number of oxidative fibers

Since abnormal muscle function, like muscular dystrophy or atrophy, could affect exercise performance we analyzed muscle structure. Histological analysis of the gastrocnemius, a major hind leg muscle in the mice, was performed. Hematoxylin eosin staining and Gomori trichrome staining of the muscles did not indicate any sign of muscular atrophy or dystrophy in the TERC$^{-/-}$ animals (data not shown). To determine the oxidative state of the fibers we performed a NADH-TR staining, which stains the high oxidative rich fibers. Differences were visible in the number of NADH-TR positive fibers in the sedentary G3

Table 4. Left ventricular measurements after four weeks of exercise in WT, TERC$^{-/-}$ G2 and TERC$^{-/-}$ G3 mice.

	WT nr (n=3)		WT vwr (n=4)		G2 nr (n=3)		G2 vwr (n=3)		G3 nr (n=3)		G3 vwr (n=4)	
animal weight (gr)	24.2	±2.4	26.2	±2.0	22.7	±2.0	22.1	±2.1	19.6	±0.2	18.9*	±1.7
heart weight (mg)	156.0	±6.1	154.3	±14.0	128.5	±23.1	148.8	±14.5	122.0	±12.9	124.8	±16.3
maximum pressure (mmHg)	76.4	±3.3	82.6	±5.4	81.2	±1.0	75.6	±3.2	81.2	±4.1	78.1	±2.4
minimum pressure (mmHg)	47.5	±4.6	56.2	±3.1	53.6	±2.5	52.5	±3.4	55.2	±5.0	51.3	±3.6
mean aortic pressure	57.2	±4.2	65.0	±3.7	62.8	±2.0	60.2	±3.3	63.9	±4.6	60.2	±3.1
ANP (relative expression)	0.47	±0.18	0.46	±0.14	0.45	±0.07	0.66	±0.42	0.23	±0.07	0.30	±0.11

Data presented as means ±SE *P<0.05 versus WT.
Abbreviations: nr: not running. vwr: voluntary wheel running, ANP: atrial natriuretic peptide.

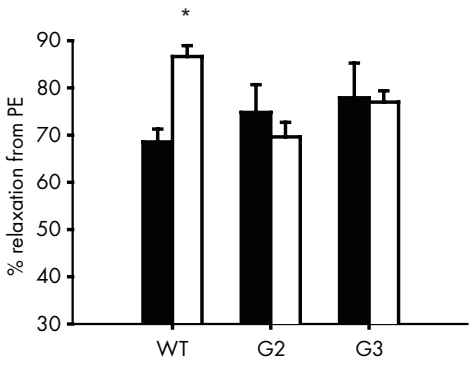

Figure 4. Endothelial function measurements by small wire myograph. Maximal vasodillatation of thoracic aorta by Ach after PE (10-4 mol/L) pre-contraction. Graph shows the difference in relaxation of the different groups on 10-4.5 mol/L Ach. Data expressed as mean ±SE, black bars represent non-running animals, white bars represent voluntary wheel running animals. * P <0.05 compared to WT non-running mice.

TERC$^{-/-}$ animals, which were less abundant as compared to WT and TERC$^{-/-}$ G2 animals. (Figure 5A-B) In the running group of G3 TERC$^{-/-}$ animals, this difference was, however, normalized to levels comparable to WT and G2 TERC$^{-/-}$ (Figure 5A-B).

Discussion

Here we investigated the effects of voluntary exercise on cardiovascular function in telomerase deficient mice. The most striking observation was the reduced exercise performance of the TERC$^{-/-}$ mice in comparison to WT mice. In order to explain this difference we measured multiple functional cardiovascular parameters, including echocardiograph, and *in vitro* vascular function measurements. Only endothelial relaxation was markedly different between WT and TERC$^{-/-}$ mice after running. WT animals showed improved vascular relaxation after running, whereas this improvement was absent in TERC$^{-/-}$ animals. In addition we observed some change in the number of oxidative fibers in the musculature of TERC$^{-/-}$ G3 sedentary animals, and this was normalized in the TERC$^{-/-}$ G3 running animals.

During the four weeks of voluntary exercise there was a significant difference in running wheel performance between TERC$^{-/-}$ animals and WT mice. The TERC$^{-/-}$ mice ran shorter distances and also spent less time in the running wheel. TERC$^{-/-}$ mice were somewhat smaller and had a reduced body weight compared to WT mice. Since Dohm et al describe that they did not find any evidence that body mass was a covariate that influenced performance[20], we believe it is unlikely that size differences could explain performance differences.

Running wheel performance of TERC$^{-/-}$ G3 mice was less than TERC$^{-/-}$ G2 mice and

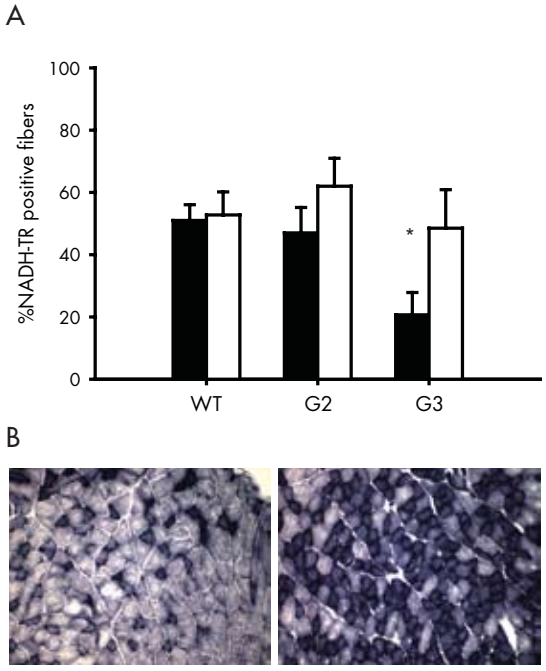

Figure 5. Oxidative fiber capacity in gastrocnemius in sedentary and voluntary wheel running mice. A. Percentage NADH-TR positive fibers in sedentary and running WT and TERC$^{-/-}$ mice. Data expressed as mean ±SE, black bars represent non-running animals, white bars represent voluntary wheel running animals. * P<0.05 compared to WT sedentary animals. B. NADH-TR staining in TERC$^{-/-}$ G3 mice. Left picture is from sedentary TERC$^{-/-}$ G3 mice, right picture is from voluntary running TERC$^{-/-}$ G3 mice. Fibers with a higher oxidative capacity are stained darker.

although the differences were not significantly different G3 mice tended to perform worse on all measured exercise parameters. As expected, G3 animals have shorter telomeres compared to G2 and WT animals. This suggests that the progressive worsening of exercise performance is associated with the shortening of telomeres and not telomerase activity per se. Werner et al. described that voluntary wheel running performance of G2 TERT$^{-/-}$ mice was comparable to WT control mice.[11, 12] In contrast to our TERC$^{-/-}$ mice, which lack the RNA component of the telomerase enzyme[18], the TERT$^{-/-}$ mice of Werner lack the catalytic subunit of telomerase.[21] Both animal models therefore lack telomerase activity and although the relative telomere shortening between both models has never been directly compared, it can be expected that this is similar. The RNA component of telomerase might have additional functions and together with telomere shortening it results in a decreased exercise activity. Gene array analysis of WT and G1 generations of TERC$^{-/-}$ and TERT$^{-/-}$ animals did, however, not reveal any expression differences[22], which suggests no major differences in TERT$^{-/-}$ and TERC$^{-/-}$ function. As it has been described that exercise performance is also influenced by the genetic background of inbred strains it is of importance to note that this might also explain some of the differences found between TERC$^{-/-}$ and TERT$^{-/-}$ mice.[23, 24] Alternatively, we cannot exclude that differences in housing conditions between our study and previous studies

might have attributed to the differences between TERC$^{-/-}$ and TERT$^{-/-}$ mice and therefore, in future studies it will be important to compare TERC$^{-/-}$ and TERT$^{-/-}$ strains directly with each other. In addition for future experiments it would be interesting to test TERC$^{-/-}$ and TERT$^{-/-}$ mice in non-voluntary exercise models and also measure maximum force generated during exercise and monitor exercise patterns during the day, to get a better understanding of the decreased exercise phenotype we observed in TERC$^{-/-}$.

Although some studies describe that a relative short period of exercise is sufficient to increase cardiac hypertrophy[19, 25], we did not notice a change in cardiac hypertrophy in any of the animals after four weeks of exercise, which is in line with the studies of Werner et al. and Zahn et al. who also noticed no signs of cardiac remodeling.[11, 26] There was no change in the heart weight to body weight ratio and we did not observe an increase in ANP expression or blood pressure at sacrifice. Although we did not observe differences in cardiac parameters, we did observe vascular effects. Exercise improved endothelial acetylcholine induced maximal vasodilatation in the WT mice, which is in line with previous studies.[12, 27] Interestingly, however, in the TERC$^{-/-}$ mice there was no improvement of the endothelial relaxation after exercise. We cannot exclude that this is a resultant of less exercise performed by these TERC$^{-/-}$ animals. However, there was a similar drop in rest heart rate in WT and TERC$^{-/-}$ animals after exercise, suggesting that the exercise induced a physiological effect.

In old mice, exercise performance is also decreased compared to young animals, but interestingly, voluntary exercise is sufficient to improve endothelial vasodilatation to the same levels as young mice.[27] Strikingly, TERT$^{-/-}$ mice also show an improvement in endothelial function after exercise[12], this is different to our results in TERC$^{-/-}$ mice and could suggest that the potential improvement of endothelial function is essential for exercise performance.

Endurance exercise is associated with a number of endurance-related muscle adaptations, including the heart muscle[19, 28-30] and changes in the skeletal muscles where exercise increases the slow twitching MHC-type I or IIa muscular fibers, fiber types with a high oxidative capacity. The increase of high oxidative fibers is at the cost of the high glycemic but low oxidative MHC-type fibers especially IIb fibers.[19, 31-34] Voluntary exercise models in rodents have shown that these adaptations occur in the tibialis anterior, soleus and gastrocnemius of exercised animals.[19, 34] In our study we determined NADH-TR percentage in the gastrocnemius, a staining that is indicative of the oxidative state of the muscle fiber. In the muscle fibers that are preferential for exercise, MHC-type I and MHC-type IIa fibers, NADH-TR levels are higher. In our WT running mice we did not see a change in oxidative fibers in the gastrocnemius. Absence of a change in oxidative fibers in the gastrocnemius was also reported by Allen et al.[19], in a comparable 4 week exercise study. We did, however, observe a clear decrease of oxidative fibers in sedentary G3 mice, and although these animals performed less exercise than the other groups, exercise increased the oxidative capacity of the fibers to the same levels as WT and G2 animals. The low oxidative fiber levels in the

sedentary G3 animals might be a result of the apparent lower activity of these animals as compared to the other sedentary animals. The musculature in TERC$^{-/-}$ G3 mice has, however, still the capacity to increase their oxidative capacity.

The most striking finding in this study was the difference in exercise performance of the TERC$^{-/-}$ mice in comparison to WT mice. Although we did not measure a single apparent factor that on its own might explain this difference, it might be a cumulative effect of multiple small changes between WT and TERC$^{-/-}$ mice. Only a limited number of parameters were analyzed and we cannot exclude the possibility that other factors such as potential glycemic control, food intake or neurological differences could have more profound effects on exercise performance. Our investigations do reveal that no major cardiac abnormalities are present in TERC$^{-/-}$ animals up to generation 3.

Acknowledgments

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Chapter 4

Bradykinin protects against oxidative stress-induced endothelial cell senescence

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Abstract

Premature aging (senescence) of endothelial cells might play an important role in the development and progression of hypertension and atherosclerosis. We hypothesized that bradykinin, a hormone that mediates vasoprotective effects of ACE inhibitors, protects endothelial cells from oxidative stress-induced senescence.

Bradykinin treatment (0.001 nmol/L – 1 nmol/L) dose-dependently decreased senescence induced by 25 μmol/L H_2O_2 in cultured bovine aortic endothelial cells as witnessed by a complete inhibition of increased senescent cell numbers and a 34% reduction of the levels of the senescence-associated cell cycle protein p21. As H_2O_2 induces senescence through superoxide-induced DNA damage, single cell DNA damage was measured by comet assay. Bradykinin reduced DNA damage to control levels. The protective effect of bradykinin also resulted in a significant increase in migration of H_2O_2 treated BAEC in an *in vitro* endothelial injury model, or "scratch" assay. The protective effect of bradykinin was abolished by the bradykinin B2 receptor antagonist HOE-140 and nitric oxide production inhibitor L-NMMA. Therefore, we conclude that bradykinin protects endothelial cells from superoxide-induced senescence through bradykinin B2-receptor- and nitric oxide-mediated inhibition of DNA damage.

Introduction

Aging-related vascular dysfunction is believed to be an important contributor to vascular pathogenesis and a worsened prognosis.[1, 2] Hypertension, one of the main risk factors for a number of cardiovascular diseases, is characterized by an accelerated development of age-related endothelial dilator dysfunction.[3] Therefore, the development of therapeutic strategies directed against age-related endothelial function decline will be an important step toward prevention and treatment of age-related vascular disease such as hypertension and coronary artery disease.

Endothelial cell senescence is a cyclin-dependent kinase inhibitor 1A (p21) proliferative arrest[4] that can be induced by DNA damage caused by exposure to reactive oxygen species (ROS).[5] There are several reasons to assume that endothelial cell senescence is involved in vascular pathogenesis. Senescent endothelial cells show deleterious functional changes (e.g., decreased release of endothelial-derived relaxing factors, increased release of endothelial-derived constricting factors and increased production of molecules that are associated with inflammation).[6, 7] These features are reminiscent of the aberrant endothelial changes observed in aging and atherosclerosis.[8] Moreover, an increase of senescent endothelial cells and senescence markers is observed in the vasculature of atherosclerotic patients and in aged rodents with endothelial dysfunction. [8, 9]

Angiotensin-converting enzyme (ACE) inhibitors effectively protect against the progression of chronic atherosclerotic disease, endothelial dysfunction, and age-related vasodilator dysfunction.[10, 11] The therapeutic effect ACE inhibitors involves a multitude of mechanisms including an increase of bradykinin (BK) levels.[12, 13] BK is a vasodilator hormone which exerts its vasomotor functions through the increase of the production and release of endothelial-derived relaxing factor[14], which takes place through the stimulation of endothelial bradykinin B2 receptors. However, the mere stimulation of vasorelaxation is a poor explanation of how BK would contribute to the improved prognosis that is brought about by ACE inhibitor treatment.

Interestingly, endothelial senescence can be prevented by increasing nitric oxide (NO)[15], one of the main relaxing factors stimulated by BK. Moreover, aging is associated with a decreased availability of cardiac bradykinin B2 receptors[16] and in some occasions with a blunted endothelium-dependent vasodilator response to BK.[17, 18] In addition, combined B2 receptor knock-out and diabetes in mice leads to an accelerated aging phenotype which is coupled to an increase of oxidative stress and an increase in ACE enzyme levels.[19] Therefore, the protective effect of BK might also relate to prevention of cellular senescence, in particular of the endothelium. Hence, we evaluated the effects of BK pretreatment on endothelial cell senescence and ROS-induced DNA damage and investigated the role of B2 receptors and NO.

Materials and methods

Cell cultures

Primary bovine aorta endothelial cells (BAEC, Tebu-Bio, Heerhugowaard, the Netherlands) were cultured in DMEM (Gibco, Breda, the Netherlands), supplemented with 10% Fetal Bovine Serum (PAA laboratories, Cölbe, Germany), 100 units/ml of penicillin and 100 µg/ml streptomycin (Gibco) in a humidified incubator at 37°C and 5% CO_2. When cells reached confluence, they were sub-cultured in a 1:10 ratio. Experiments were conducted on cells with a passage number between 3 and 9.

ROS induced senescence and pharmacological studies

BAEC cells, 24 hours after seeding 3000 cells per well in 24- wells clusters, were exposed to different concentrations of H_2O_2 (Merck, Amsterdam, the Netherlands), to induce DNA damage and senescence. After 1 hour the medium was replaced with normal medium. Various concentrations of BK (Sigma-Aldrich, Zwijndrecht, the Netherlands) were administered 30 minutes before induction of senescence. The B2 antagonist, HOE-140 (Sigma-Aldrich), and the NO inhibitor L-NMMA (N_ω-Methyl-L-arginine acetate salt (Sigma-Aldrich), were added 5 minutes before BK treatment after which BK was added without replacing the medium. At least 3 separate experiments were performed per treatment.

Evaluation of the number of senescent cells

To determine the number of senescent cells, senescence-associated-β-galactosidase (sa-β-gal) staining was performed.[20] Cells were washed in PBS and fixated in 2% formaldehyde and 0.2% glutaraldehyde for 5 minutes at room temperature, washed and incubated for 18 hours at 37°C with sa-β-gal staining solution (150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_2Fe(CN)_6$), 40 mmol/L citric acid / sodium phosphate dibasic at pH 6.0, containing 1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-D-galactoside). Light microscopic pictures were taken on an inverted microscope (Zeiss Axiovert 135M) with a 200x magnification and the number of senescent cells was counted per microscopic field. In each well 5 random fields were evaluated.

P21 protein detection

Cultured cells were lysated with 1x SDS sample buffer (62.5mmol/L Tris-HCL, 2% SDS, 10% glycerol, 50mol/L DTT, 0.01% bromophenol blue). Lysates were analyzed by standard Western blotting techniques under denaturing conditions. Membranes were incubated overnight at 4 °C with the primary p21 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) at a 1:200 dilution. Membranes were reprobed with β-actin antibody (Sigma-Aldrich) and incubated for 1 hour at room temperature at a 1:10000 dilution. Signals were

detected by the ECL-detection method and quantified by densitometry. P21 protein levels were corrected for β-actin and results are expressed as a percentage off p21 levels of BAEC at passage 31, of which cell protein extracts were run in parallel for each blot.

Apoptosis

Apoptosis was determined with the Caspase-Glo 3/7 Assay (Promega, Leiden, the Netherlands) 24 hours after treatment.

In 96-well plates, 50 µl sample was mixed gently for 30 s with 50 µl of Caspase-Glo 3/7 reagent and incubated for 2 h at room temperature. Apoptosis was determined by luminescence of the samples measured using a Victor Wallac Multilabel Counter 1420.

DNA damage assay

DNA damage was determined by comet single cell electrophoresis assay. BAEC cells were treated as described before with H_2O_2 and BK, 24 hours after treatment cells DNA damage was determined with CometAssay (Trevigen, Gaithersburg, USA). Cells were harvested and approximately 700 cells were placed on a CometSlide in LMAgarose. Cells were lysated one hour at 4°C in Lysis Solution. Denaturation was performed for 30 minutes in 0.3 mol/L, 1 mmol/L EDTA pH>13. Electrophoresis was done in 1x TBE (tris-borate-EDTA, pH 8.3). Comet slides were stained with SYBR Green and photos were taken at a 100x magnification. Pictures were analyzed with CASP 1.2.2 software.[21] Experiments were repeated three times and in total more than 248 cells were analyzed per treatment group.

In vitro endothelial injury model

To test the functional consequences of senescence induced by H_2O_2, the *in vitro* scratch injury model was used and adapted for BAEC cells. Cells were seeded in a 96-wells cluster at a density of 2000 cells per well. Cells were treated 24 hours after seeding. 24 hours after treatment a thin line scratch between the cells was created with a 10 µL pipette tip (Greiner Bio-One, Alphen aan den Rijn, the Netherlands). After scratching, the wells were washed with PBS and fresh medium was added. 7 hours later the wells were fixed for 10 minutes with 2% formaldehyde and 0.2% glutaraldehyde. Each treatment was conducted in at least 7 wells, divided over two different experiments. Two pictures were taken per well and the width of the scratch was measured at 4 points per picture with Image-Pro Plus (Media Cybernetics, Bethesda USA) and the means were calculated. Then the means of the 2 pictures were calculated to obtain n=1.

Statistics

Data represent mean values +/- standard errors of the means (SEM). Comparisons between groups were done by one way ANOVA with Bonferroni or Dunnett's post-hoc

corrections for multiple comparisons. P values < 0.05 were considered a significant difference.

Results

Induction of senescence

To optimally study the effects BK, a protocol for ROS-induced senescence was designed in such a way the outcome is not confounded by apoptosis. Therefore, BAEC were exposed to various concentrations of H_2O_2 for 1 hour and subsequently cultured for 3 days, after which senescence and apoptosis was measured. H_2O_2 dose dependently increased the number of senescent cells leading to significant increases at concentrations of 30 and 100 µmol/L (Figure 1A and 1B). Exposure to concentrations of H_2O_2 of 100 and higher led to apoptosis, whereas 25 µmol/L H_2O_2 was without effect on caspase-3 activity (Figure 1C).

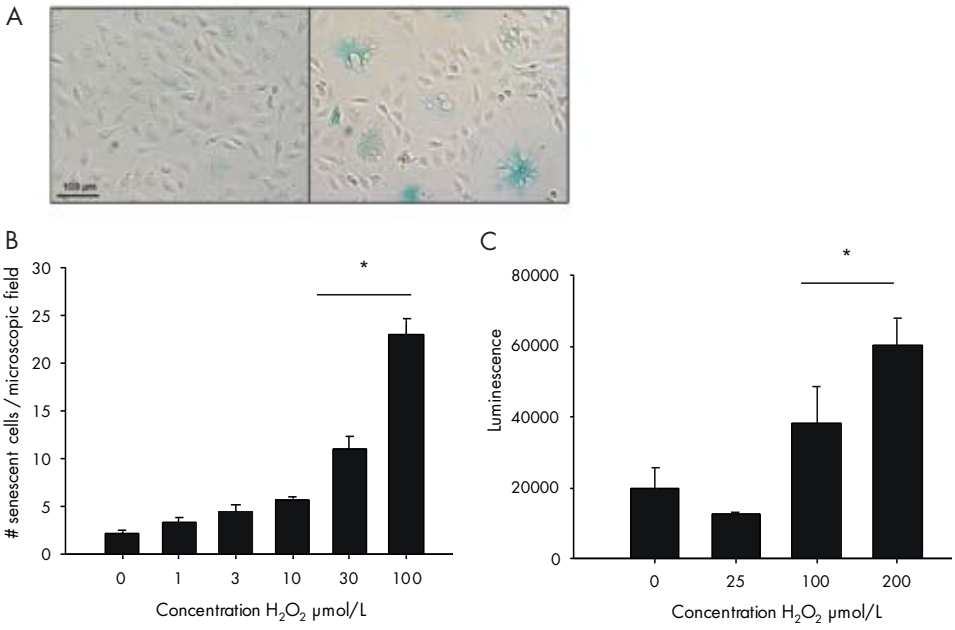

Figure 1. Low dose of H_2O_2 induces senescence in BAEC without causing apoptosis. A. Representative pictures of SA-β-galactosidase-stained BAEC cells. Left panel: control (no H_2O_2), right panel: 3 days after stimulation with 25 µmol/L H_2O_2 for 1 hour. B. Number of SA-β-galactosidase-positive cells per microscopic field 3 days after a 1-hour H_2O_2 treatment. Bars represent mean +/- SEMs (*: p<0.05 vs. control, n=6 experiments). C. Caspase-3 activity, measured as luminescence, in BAEC 24 hours after treatment with H_2O_2 for 1 hour. Bars represent means +/- SEMs (*: p<0.05 vs. control, n=3 experiments).

Further experiments were therefore performed with 25 µmol/L H_2O_2. This approach warrants that the cell counting procedure and the measured effects and DNA damage is not confounded by apoptosis-related changes.

BK protects against endothelial senescence through B2 receptors

To test the effect of BK on senescence induced by 25 µmol/L H_2O_2, BAEC cells were pretreated with different concentrations of BK (Figure 2A). BK caused a dose-dependent decrease in the number of senescent cells, reaching a maximum effect at a concentration of 1 nmol/L which fully inhibited senescence. There was no effect of BK on the cells that were not treated with H_2O_2.

As a secondary variable to assess senescence, p21 expression was measured by western blot analysis. P21 is an important mediator of endothelial cell senescence.[4] P21 levels were increased 24h after treatment with 25 µmol/L H_2O_2. BK (1 nmol/L) fully prevented the change in p21 levels, thus confirming the effect observed when counting senescent cells (Figure 2B).

Induction of cell proliferation might confound the effects observed on the p21 levels. Therefore, we tested the effect of 1 nmol/L BK on proliferation of BAEC, and found no proliferative effect of BK after 24 and 48 h (data not shown).

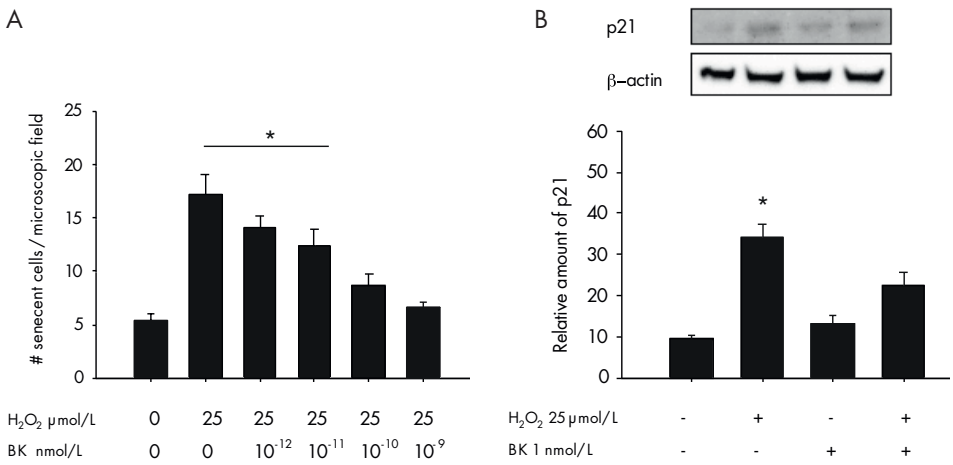

Figure 2. Effects of bradykinin pre-treatment, given 30 minutes in advance, on H_2O_2-induced senescence of BAEC (passage 3 to 9). A. Number of senescent cells 3 days after treatment. Bars represent mean values +/- SEMs (* $p<0.05$ vs. control, n=6 experiments) B. P21 protein levels 24 hours after treatment. P21 levels were corrected for β-actin levels and expressed as % of p21 levels in old BAEC from passage 31. (Bars represent mean values +/- SEMs (*: $p<0.05$ vs. to control, n=6 experiments).

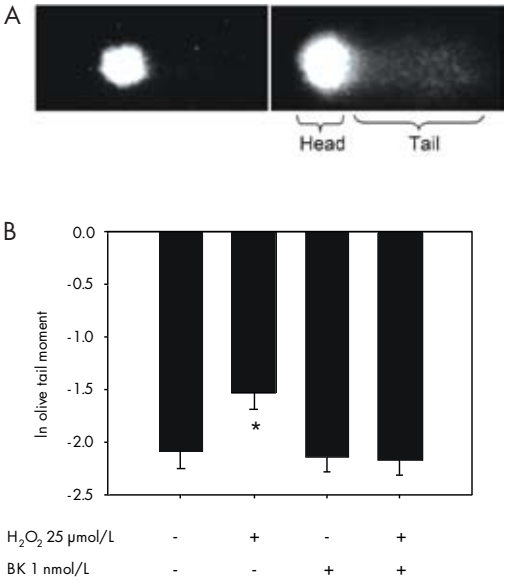

Figure 3. DNA damage in BAEC 24 hours after treatment, measured by comet assay.
A. Example of the BAEC DNA single cell electrophoresis. Left panel: control (no H_2O_2), right panel: 1 day after stimulation with 25 µmol/L H_2O_2 for 1 hour. Olive tail moment is calculated as % DNA in the tail x distance to centre of gravity of tail.
B. Olive tail moment, expressed as % DNA in the tail x distance to centre of gravity of tail.The DNA damage is represented as the natural log of the olive tail moment. Bars represent means +/- SEMs), (*: $p<0.05$ vs. control, n=3 experiments).

BK protects against DNA damage

Protection against senescence can take place in a number of ways, one of which is protection against DNA damage. Therefore, we measured the effect of 1 nmol/L BK DNA damage caused by 25 µmol/L H_2O_2 by determining the olive tail moment in a comet assay 24 hours after treatment. H_2O_2 increased the olive tail moment (Figure 3), thus indicating an increase in DNA damage. BK prevented an increase of DNA damage, whilst being without effect in cells that were not treated with H_2O_2.

BK protects cells from senescence-induced impaired cell migration

To test if senescence and the protective effect of BK altered endothelial cell function, the scratch assay, an accepted method to test the ability of endothelial cells to migrate[22], was performed (Figure 4). Cells treated with 25 µmol/L H_2O_2 showed a reduced ability to migrate, as compared to the other groups. BK fully prevented the H_2O_2 effect, without displaying any effect in cells that were not treated with H_2O_2.

BK protection against endothelial cell senescence depends on BK B2 receptor mediated NO release

BK exerts many of its effects in adult tissues through the BK B2 receptor.[23] To check if the protective effect against senescence is also dependent on the BK B2 receptor we studied the effect of the BK B2 receptor antagonist HOE-140 (1 µmol/L) on reduction of senescent cells by BK. HOE-140 fully antagonized the protective BK effect whilst being without effect

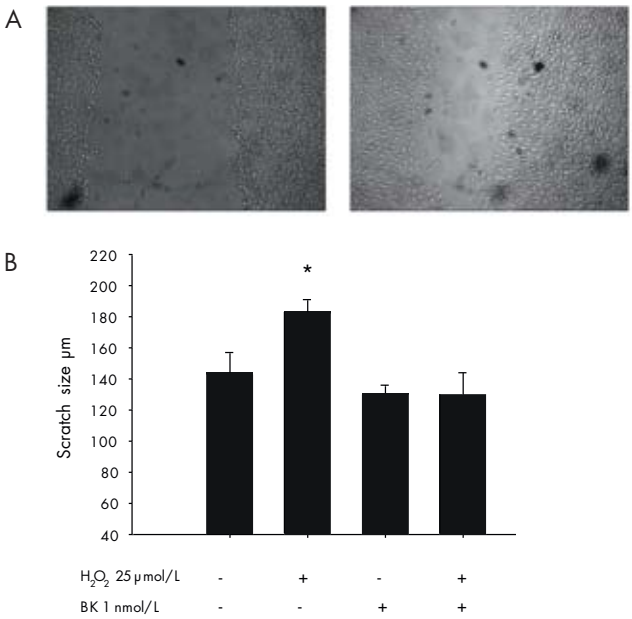

Figure 4. Cell scratch assay with BAEC treated with H_2O_2 25 µmol/L, BK 1nmol/L or the combination. A. Example of cell scratch assay. Left panel, BAEC immediately after making the scratch. Right panel, scratch size after 7 hours in untreated BAEC. B. Scratch size in different groups 24 hours after treatment. Bars represent scratch width in µm, 7 hours after scratching +/- SEMs (*: p<0.05, n=7 experiments).

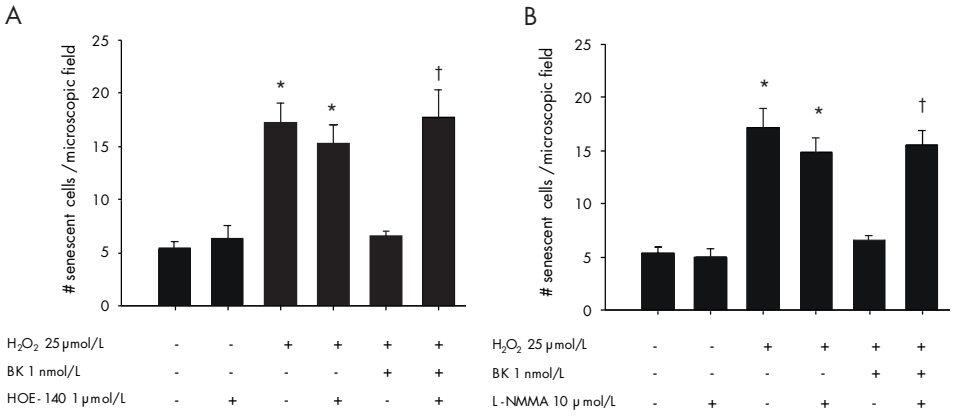

Figure 5. The role of BK B2 receptor and NO in the protective effect of BK against senescence in BAEC. Senescence was measured as the number of SA-β-galactosidase-positive cells 3 days after treatment with H_2O_2. A. Effect of BK-B2 receptor antagonist HOE-140. B. Effect of NO inhibitor L-NMMA. Bars represent means +/- SEMs n=6 (*: p<0.05 vs. control, † p<0.05 vs. BK 1 nmol/L).

in cells that were not treated with H_2O_2 (Figure 5A). As stimulation of this receptor leads to the release of NO, we investigated the effect of blockade of NO production by L-NMMA (10 µmol/L). L-NMMA inhibited the protective effect of BK (Figure 5B) and had no effect in the absence of BK. Therefore, BK protects against senescence through stimulation of BK B2 receptor-mediated NO release.

Discussion

We show here that BK protects against ROS-induced DNA damage and endothelial cell senescence through BK B2 receptor-mediated NO release. The protective effect of BK is associated with a decrease of p21, a cyclin-dependent kinase 1A inhibitor that is normally activated by DNA damage. BK also prevents suppression of endothelial cell migration by ROS, thus demonstrating the functional importance of the protective effect of BK. These observations are in line with studies that explore the role of the kinin system in aging. Previously it has been shown that BK B2 receptor knock-out mice display a slightly accelerated aging phenotype, as observed by increased alopecia, skin atrophy, kyphosis, osteoporosis, lipofuscin accumulation in renal proximal tubule and testicular Leydig cells, and apoptosis in the testis and intestine. Importantly, this aging phenotype is strongly promoted when BK B2 receptor knockout is combined with diabetes.[19] This underlines the importance of the coexistence of a risk factor that adds metabolic, oxidative stress during the process of premature aging. This process is apparently counterregulated by the kinin system, not only in the cardiovascular system, but also in a broader perspective, and this protective role of the kinin system seems to depend on protection against ROS-induced DNA damage, as witnessed by our present study.

This study shows that stimulation of a G-protein coupled receptor that leads to eNOS activation can protect against endothelial senescence. Although we are not aware of any other study showing a similar role of G-protein coupled receptors, the contrary effect, i.e. induction of senescence, can result from stimulation of angiotensin II type 1 receptors in vascular smooth muscle cells[24] and endothelial progenitor cells.[25] Therefore, these previous studies combined with our present results suggest that intervention through RAS inhibition can be dually protective: on the one hand angiotensin II type 1 receptor blockade prevents ROS-induced senescence, and on the other hand ACE inhibition protects against ROS-induced senescence through stimulation of BK B2 receptor signaling. ACE inhibitors promote BK B2 receptor signaling in two ways. The first possibility is an increase of BK levels due to decreased metabolism of this peptide hormone.[12] The second way is potentiation of the BK response through local interactions in endothelial cells that lead to an increased number of BK B2 receptor in their affinity state, both in absolute receptor numbers (measured as

binding sites) as well as relative to the number of receptors in the low affinity state.[26, 27] ACE inhibitors might also decrease formation of angiotensin II. However, during ACE inhibition angiotensin II levels are rapidly restored through the so-called "ACE escape", a bypass created by alternative metabolic pathways to restore angiotensin II formation.[28, 29] Vascular angiotensin II responsiveness is associated with an increased risk of future cardiovascular events.[30] This underscores the potential importance of BK-mediated inhibition of senescence as a mechanism that counterbalances the deleterious effects of angiotensin II signaling. The interaction between BK and angiotensin II signaling pathways in the control of cellular senescence needs to be elucidated, and requires further confirmation in *in vivo* models and patient studies.

The observation in the present study that BK B2 receptor signaling shows a relationship with the function of cell cycle proteins such as p21 is not unique. It has been shown that the gene encoding for the BK B2 receptor contains a binding motive for p53[31], a transcription factor that is activated by DNA damage and that plays an important role in regulation of p21 and cellular senescence.[32] Forced overexpression of p53 in HeLa cells activated the rat BK B2 receptor promoter region in reporter constructs. Our present data complement this observation in HeLa cells and, taken together, these studies generate the following, simple concept: DNA damage leads to p53 activation, which in turn causes upregulation of BK B2 receptors. As a result BK B2 receptor activity increases, serving as an endogenous protective feedback mechanism against further ROS-induced DNA damage, and thus reducing p21-mediated cellular senescence and loss of cellular function. This concept becomes even more compelling if one considers that cardiac BK B2 receptors decrease with age. This means that aging itself leads to a decline of the BK-mediated protection against cellular aging, which would lead to a downward spiral of loss of cell function, especially in situations where oxidative stress is increased. This is underlined by the finding that combined BK B2 receptor knockout and diabetes leads to a progeoid phenotype in mice.[19] It is unclear how BK B2 receptor signaling leads to protection against DNA damage. The fact that NO is involved gives directions towards a possible explanation, which is improved DNA repair. NO has been described to protect against replicative senescence whilst NO blockade promotes senescence, effects that were associated with telomeric DNA repair by telomerase.[15, 33] Other studies contradict this effect of NO on replicative senescence.[34] Nevertheless, we here show that NO is also involved in ROS-induced senescence and mediates protection against the degradation of DNA on a more global chromosomal DNA scale, which might involve various DNA repair systems such as double-strand break repair mechanism that were shown to mediate the protective effect of statins against vascular smooth muscle cell senescence.[35] Alternatively, BK could activate anti-oxidant pathways. This intriguing question opens an opportunity to further explore and understand the importance of the kinin system and the mechanisms of ACE inhibition.

Perspectives

Our data show that BK can protect endothelial cells from cellular aging in an *in vitro* model. When combined with the findings of other studies, our findings contribute to a novel paradigm that hypothesizes that the kinin system plays an important role in the control of progressive loss of function due to the continual DNA damage that occurs during aging. The maintenance of a proper function of the kinin system by pharmacotherapy, such as ACE inhibition, in the aging population seems crucial. Since the activity of the kinin system decreases with aging, supplemental suppression of oxidant stress, e.g. by AT1 receptor antagonist therapy, improvement of DNA repair, e.g. by statins[35], and proper control of diabetes and a healthy life style appears to be indispensable. Furthermore, it is worthwhile to explore the reasons for age-related decrease of kinin system activity and seek preventive measures. Organ and cell type specificity, and interactions with or effects of other vasoactive peptides, such as angiotensins, will need to be addressed. This will help to identify new drug targets as well as provide further explanations with respect to the beneficial or unbeneficial effects of already applied drug therapies in cardiovascular disease. Last but not least, the paradigm presented here needs to be confirmed by clinical data.

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Chapter 5

Glucagon-Like Peptide 1 prevents ROS induced endothelial cell senescence through the activation of protein kinase A

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Abstract

Objective

Endothelial cell senescence is an important contributor to vascular aging and is increased under diabetic conditions. Here we investigated whether the anti-diabetic hormone, glucagon-like peptide 1 (GLP-1), could prevent oxidative stress-induced cellular senescence in endothelial cells.

Methods and results

In Zucker diabetic fatty (ZDF) rats, a significant 2-fold higher level of vascular senescence was observed with control lean rats. Dipeptidyl-peptidase 4 (DPP-4) inhibition significantly increased GLP-1 levels in these animals and reduced senescence almost to lean animal levels. *In vitro* studies with human umbilical vein endothelial cells (HUVEC) cells showed that GLP-1 had a direct protective effect on oxidative stress (H_2O_2)-induced senescence and was able to attenuate oxidative stress-induced DNA damage and cellular senescence. The GLP-1 analogue exendin-4 provided similar results, whereas exendin fragment 9-39, a GLP-1 receptor antagonist, abolished this effect. Intracellular signaling by the phosphoinositide 3-kinase (PI3K)/Akt survival pathway did not appear to be involved. Further analysis revealed that GLP-1 activates the cAMP response element-binding (CREB) transcription factor in a cAMP/protein kinase A (PKA)-dependent manner, and inhibition of the cAMP/PKA pathway abolished the GLP-1 protective effect. Expression analysis revealed that GLP-1 can induce the oxidative defense genes *HO-1* and *NQO1*.

Conclusions

Dipeptidyl-peptidase 4 inhibition protects against vascular senescence in a diabetic rat model. *In vitro* studies with human umbilical vein endothelial cells showed that reactive oxygen species-induced senescence was attenuated by GLP-1 in a receptor-dependent manner involving downstream PKA signaling and induction of anti-oxidant genes.

Introduction

Vascular aging is an important contributor to the progression of diabetes and cardiovascular diseases and is associated with a worsened prognosis.[1, 2] In diabetes and atherosclerosis there is a causal link between endothelial dysfunction and the progression of these diseases.[1-4] One of the main contributors is the increase in reactive oxygen species (ROS), which increases intra-cellular (DNA) damage and ultimately can result in the onset of apoptosis or the induction of cellular senescence.[5, 6] Increased levels of cellular senescence have been observed in the vasculature of patients with coronary artery disease[7, 8] and in tubular compartments of patients with diabetic nephropathy.[9] In Zucker diabetic fatty (ZDF) rats, decreased aortic vasodilatation was accompanied by an increase in senescent positive endothelial cells in the aorta.[10] The ZDF rat develops obesity-related diabetes associated with increased levels of free fatty acids and ROS in its vasculature.[11, 12] Treatment of these animals with the anti-oxidant drug ebselen prevented vascular senescence and reduced vasculopathy.[10]

In the treatment of type 2 diabetes, the glucagon-like peptide 1 (GLP-1) receptor is a novel target. GLP-1 is proglucagon-derived hormone produced by the intestinal L-cells in response to food intake and has a profound effect on glycemic control.[13] GLP-1 reduces gastric emptying, and reduces glucagon secretion from the α-cells. Most pronounced are its effects on the β-cells where GLP-1 stimulates insulin production, increases proliferation and can reduce β-cell apoptosis.[14] *In vivo*, active GLP-1 is rapidly degraded by dipeptidyl-peptidase 4 (DPP-4). Therefore, more stable GLP-1 analogues, such as exenatide, and DPP-4 inhibitors, such as vildagliptin, have been developed for the treatment of type 2 diabetes.

GLP-1 acts through the GLP-1 receptor, a G-coupled-protein receptor, which is abundantly present in the gastrointestinal tract, but has also been detected at lower levels in the nervous system, the heart, vascular smooth muscle and endothelial cells.[15-17] GLP-1 is a potent vasodilator and is associated with the improvement of endothelial function in animal models and in type 2 diabetic patients.[17, 18] Activation of the GLP-1 receptor can trigger at least 2 downstream pathways, generation of the second messenger cyclic adenosine monophosphate (cAMP) followed by activation of protein kinase A (PKA), and the indirect activation of epidermal growth factor receptor (EGFR) followed by phosphoinositide 3-kinase (PI3K) and Akt signaling.[14, 19]

We hypothesized that GLP-1 receptor activation could be involved in reducing the detrimental effects of oxidative stress and reduce cellular stress in the vasculature. Here we used an *in vivo* and *in vitro* model to investigate the effects of GLP-1 on cellular senescence.

Methods

Animal studies

All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen. Male ZDF (ZDF-Lepr^{fa}/Crl) rats and lean littermate (ZDF-Lepr^{fa}/+/Crl) rats were obtained from Charles River (Charles River, Maastricht, the Netherlands). At 10 weeks of age, ZDF animals were allocated in two groups (n=7 per group), and 1 group was treated with vildagliptin (Galvus®, Novartis Pharma AG, Basel, Switzerland) for 15 weeks at a dose of 3 mg/kg per day (via the drinking water). Untreated ZDF rats and age-matched lean controls (n=7) rats served as controls.

Senescence-associated ß-galactosidase staining

Senescence was determined by senescence-associated β-galactosidase (sa-β-galactosidase) staining.[20]

Senescence staining of aorta

Rat abdominal aorta was cleaned by removing fat and connective tissue and subsequently stained for senescence as described above. After staining, a part of the aorta was transversely cut and stored for cryo-sectioning, the rest of the aorta was cut longitudinally and pictures were taken from the aorta with a DSLR camera equipped with a 50 mm macro lens. Photos of aortas were randomized and the percentage of senescence staining was determined with ImageJ software.[21]

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from the Endothelial Cell Facility (University Medical Center Groningen, the Netherlands). HUVECs were isolated from 2 umbilical cords and prepared as previously described.[22] Cells were cultured on 1% precoated gelatin plates at 37°C under 5% CO_2. RPMI 1640 medium was supplemented with 20% FCS, 2 mmol/L $_L$-Glutamine, 5 U/mL heparin, 100 IU/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL endothelial cell growth factor extracted from bovine brain. Experiments were conducted with cell passage numbers between 2 and 6.

Premature senescence assay

Senescence was induced by adapting our previously published endothelial senescence model.[5] In short, HUVECs were seeded at 5000 cells/cm^2 on 1% gelatin-coated plates. After 24 hours medium was replaced with low-serum medium (2% fetal calf serum), followed by overnight incubation. Senescence was induced by exposing cells to 30 µmol/L

H_2O_2. After 1 hour medium was replaced with normal growth medium, and at confluence, the percentage of senescent cells was determined. Various concentrations of GLP-1, exendin-4, 8-Bromoadenosine-cAMP (8-Br-cAMP), and forskolin were administered 30 minutes before H_2O_2 exposure, and exendin (9-39), LY294002, H89, KT 5720, and NG-monomethyl-$_L$-arginine (L-NMMA) were administered 30 minutes before GLP-1 addition. Senescent staining was performed as described above. Light microscopic pictures were taken on an inverted microscope with a x10 objective, and the number of senescent cells and total cell number were counted per microscopic field. In each well, 4 random fields were evaluated. Data from multiple experiments were compared by calculating the relative amount of senescence, for which the 30 μmol/L H_2O_2 group was set to 1.00 in each separate experiment.

Protein analysis

For Western blot analysis of whole cell lysates, the following antibodies were used: anti-phosphorylated Akt (Ser473), anti phosphorylated CREB (87G3), anti-caspase-3 (8g10) (Cell Signaling Technology, Danvers, Mass), and anti-GLP-1-receptor (H55) (Santa Cruz Biotechnology, Heidelberg, Germany). For loading control, membranes were re-probed with β-actin antibody (Sigma-Aldrich), α-tubulin (Sigma-Aldrich), or GAPDH antibody (Fitzgerald Industries International, Acton, Mass). Signals were detected by ECL and quantified by densitometry (Syngene, Cambridge, United Kingdom).

Quantitative real-time polymerase chain reaction

Relative expression of *HO-1* and *NQO1* genes was determined by quantitative polymerase chain reaction (qPCR). Gene expression was determined by correcting samples for reference gene values (cyclophilin A), and values were expressed relative to the control group per experiment.

Comet assay

DNA damage was determined by the comet single cell electrophoresis assay, as described by the manufacturer (Trevigen, Gaithersburg, Md).

Statistics

Data represent mean values ±standard errors (SE). Comparisons between groups were done by 1-way ANOVA with post-hoc Dunett correction. Comparison of the sa-β-galactosidase areas in the abdominal aortas and qPCR expression was performed by Mann-Whitney U test. A probability value of less than 0.05 was considered significant.

Expanded methods can be found as supplemental material.

Results

DPP-4 inhibitor vildagliptin decreases vascular senescence in vivo

Here we investigated vascular senescence in normal "lean" animals as compared with ZDF diabetic rats. Senescence in abdominal aorta was determined with the well-established sa-β-galactosidase staining.[20] As shown in Figure 1A, staining was most pronounced in the areas of aorta side branches, in concordance with the study of Brodsky et al.[10] Determination of the amount of senescence per vessel showed a significant increase in 25-week-old ZDF animals as compared with control lean animals (Figure 1B), 4.7±1.1% versus 2.3±0.5%. Histological analysis of the stained vessels confirmed that parts of the endothelial cell layer were stained positive for sa-β-galactosidase (Figure 1C). Treatment of the ZDF animals with vildagliptin resulted in a significant reduction of DPP-4 activity (Figure 1D) and an almost 6 fold increase of GLP-1 plasma levels (Figure 1E). No changes in plasma glucose and insulin levels were observed and body weights remained constant with this treatment protocol (Supplemental Table I). The vildagliptin treatment did, however, decrease cellular senescence in

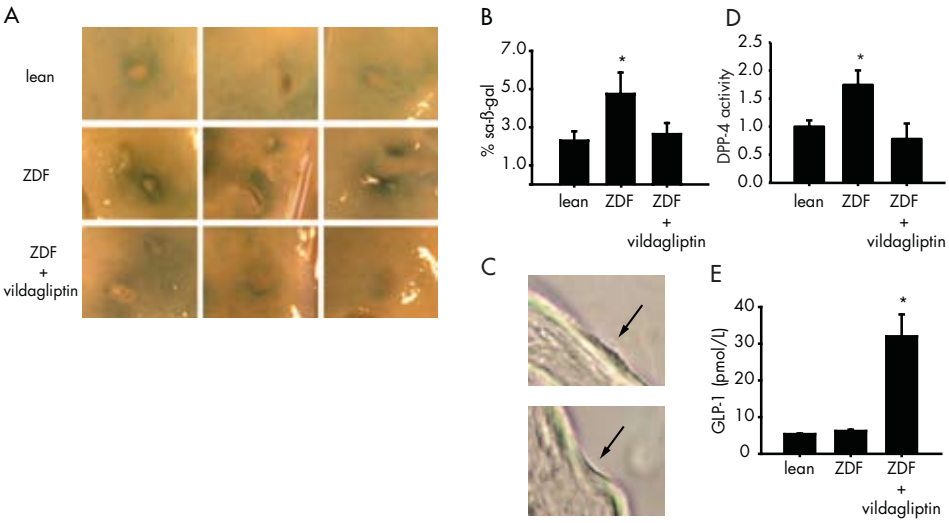

Figure 1. DPP-4 activity and senescence in ZDF animals treated with vildagliptin. A. Abdominal aortas stained with sa-β-galactosidase. Photos are representative pictures for the different treatment groups. B. Percentage senescence visible in the aortas of lean, ZDF and ZDF + vildagliptin treated animals after sa-β-galactosidase staining. Data are expressed as mean ±SE, n=7. * P<0.05 compared with the lean group. C. Examples of endothelial cells stained positive for sa-β-galactosidase in the aortas of ZDF animals. Arrows indicate positive endothelial cells. D. Relative DPP-4 activities in lean, ZDF and ZDF + vildagliptin treated rats. Data are expressed as mean ±SE. Data are from at least 4 animals per group. * P<0.05 compared with the ZDF + vildagliptin group. E. GLP-1 plasma levels in lean, ZDF and ZDF + vildagliptin treated animals. Data are expressed as mean ±SE, n=7. * P<0.05 compared with the ZDF group.

these animals, to levels almost comparable to those of lean rats (2.6±0.6% versus 2.3±0.5%) (Figure 1A and 1B). This suggests that increased GLP-1 levels by DPP-4 inhibition have a protective effect on the vasculature.

GLP-1 treatment inhibits ROS induced premature senescence

To investigate the effects of GLP-1 on endothelial senescence, we switched to the well-established HUVEC cell system, which is known to be sensitive to stress-induced cellular senescence. In HUVECs, cellular senescence was induced by oxidative stress via exposure to low concentrations of H_2O_2 (30 µmol/L), which did not induce apoptosis, as confirmed by the absence of caspase-3 cleavage under these conditions (Supplemental Figure I). This low dose, however, resulted in a significant 3.4-fold increase, (from 2.00±0.25% to 6.82±0.88%), in the number of senescent cells 3 days after exposure, as determined by sa-β-galactosidase staining (Figure 2A). Treatment of HUVECs with GLP-1 attenuated the increase of senescent cells in a dose responsive manner (Figure 2B), with a maximum of approximately 63% reduction of senescent positive cells compared with non-GLP-1 treated group at a concentration of 10 nmol/L GLP-1 (Figure 2B) (H_2O_2 group 0.99±0.08; 10 nmol/L GLP-1 + H_2O_2 group 0.37±0.03; control group 0.32±0.03).

In parallel to the sa-β-galactosidase staining also DNA damage was also investigated in HUVECs using the comet assay (Figure 3A). The tail DNA read-out showed that H_2O_2

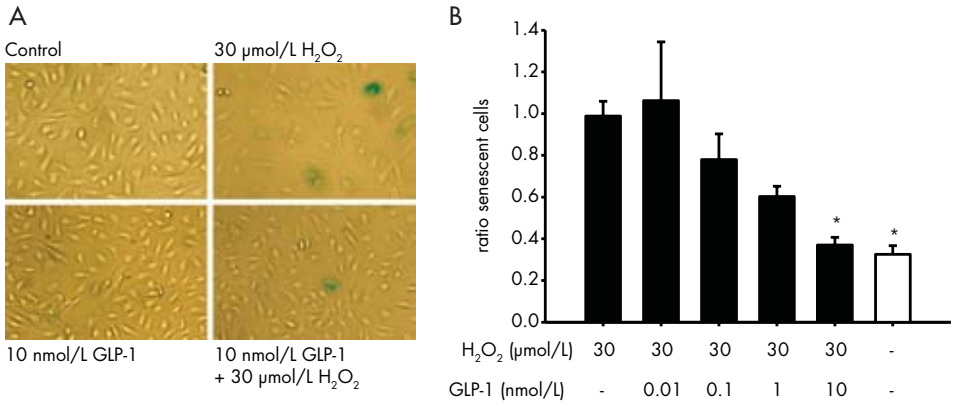

Figure 2. GLP-1 treatment protects HUVECs from stress induced senescence. A. Representative images of HUVECs stained with sa-β-galactosidase, 3 days after treatment with GLP-1, H_2O_2, or both. B. Dose response effect of GLP-1 treatment 30 minutes before cells were exposed to 30 µmol/l H_2O_2. The number of senescent cells is expressed relative to the H_2O_2 30 µmol/l group. Data are expressed as mean ±SE. Data from control, 30 µmol/l H_2O_2 and GLP-1 10 nmol/l + 30 µmol/l H_2O_2 were obtained from 7 experiments; the other data points were repeated 5 times. Open bar represents the non-treated control group. *P < 0.05 compared with the H_2O_2 group.

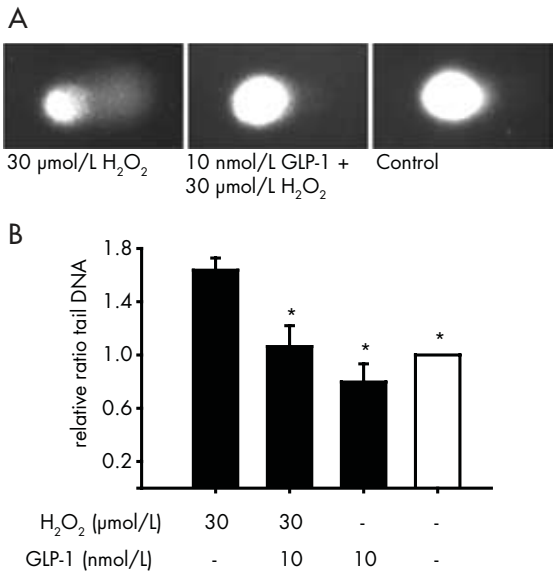

Figure 3. DNA damage in HUVECs measured by a comet assay, 24 hours after treatment. A. Panel of three examples of HUVEC DNA single-cell electrophoresis. Left, control; middle, 1 hour 30 µmol/l H_2O_2 exposure; right, treated with 10 nmol/l GLP-1 for 30 minutes followed by H_2O_2 exposure. B. Ratio of tail DNA in the different treatment groups. Ratios are expressed relative to the non-treated control group (open bar). Data are expressed as mean ±SE. Data are from 3 different experiments. *P < 0.05 compared with the H_2O_2 group.

significantly increased DNA damage by 1.6-fold relative to the control group (Figure 3B). In GLP-1 treated cells, H_2O_2 exposure induced DNA damage only by 1.1 fold. Thus, GLP-1 significantly reduced DNA damage generated by H_2O_2 as determined by the percentage tail DNA in a comet assay. Together, these findings suggest that GLP-1 treatment can reduce ROS induced DNA damage and consequently attenuate the increase in senescent HUVECs.

The protective effect of GLP-1 is receptor mediated

Western blot analysis of cell extracts showed GLP-1 receptor expression in HUVECs (Supplemental Figure II), which is in line with other studies showing endothelial expression. [15, 17] Next we used the GLP-1 analogue exendin-4 (an agonist of the GLP-1 receptor). Similar to GLP-1 treatment, exendin-4 strongly attenuated H_2O_2 induced senescence in HUVECs (Figure 4A). Co-treatment with exendin (9-39), another GLP-1 derived peptide that acts as an antagonist for the GLP-1 receptor, completely abolished the protective effect of GLP-1 on H_2O_2 induced cellular senescence (Figure 4B). These data provide evidence that the GLP-1 protective effect is GLP-1 receptor mediated.

The PI3K/Akt survival pathway is not required for the GLP-1 protective effect

In pancreatic β-cells[23] and neuronal cells[24] GLP-1 prevented apoptosis through the activation of PI3K and Akt. Although the amount of oxidative stress used in this study did not induce apoptosis in our HUVEC cell model, the PI3K/Akt signaling pathway is a general survival pathway, and we therefore tested whether this pathway could be involved

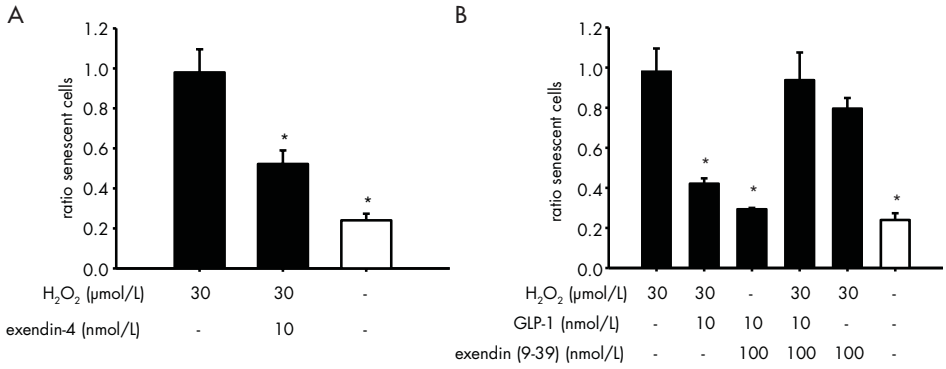

Figure 4. Effects of exendin-4 and exendin-fragment 9-39 on cellular senescence. Senescence is expressed relatively to the H_2O_2 group. A. Effect of the GLP-1 receptor analogue exendin-4 on cellular senescence. B. Effect of the GLP-1 receptor antagonist exendin (9-39). For both graphs senescence is expressed as the relative ratio to the H_2O_2 groups. Data are expressed as mean ±SE. Data were obtained from 4 experiments; the controls exendin-4 10 nmol/l and exendin fragment (9-39) + GLP-1 10 nmol/l were repeated 3 times. *P < 0.05 compared with H_2O_2 groups.

in the protective effect of GLP-1. The activity of Akt kinase was determined using an antibody against phosphorylated serine 473, the activation residue of Akt. Western blot analysis with this antibody did not reveal any change in Akt activity between control and GLP-1 treated cells in the presence of H_2O_2 (Figure 5A and 5B), indicating that GLP-1 does not activate this kinase under these conditions in HUVECs. To further rule out the involvement of this pathway, the upstream activator of Akt, PI3K, was inhibited using the small molecule LY294002 (Supplemental Figure III). As shown in Figure 5C, inhibition of PI3K by LY294002 did not result in a decrease of the protective effect of GLP-1 in HUVECs. Together these data do not support a role of the PI3K/Akt pathway in the GLP-1 mediated effect in HUVECs.

GLP-1 protective effect is cAMP/PKA dependent

The GLP-1 receptor is a G-protein coupled receptor and activates adenylate cyclase, resulting in cAMP production and downstream PKA activation. We therefore investigated the involvement of this intracellular signaling cascade in the GLP-1 protective effect. Treatment with forskolin, an adenylate cyclase activator, attenuated H_2O_2-induced cellular senescence in HUVECs (Figure 6A). Similar results were obtained with 8-Br-cAMP, a membrane permeable cAMP analogue (Supplemental Figure IV A). The effect of forskolin was dependent on the downstream cAMP effector PKA, as specific inhibition of PKA with H89 could abolish the forskolin-mediated protection. PKA inhibition by H89 was also sufficient to block the GLP-1 mediated protective effect on HUVECs (Figure 6B). H89 inhibition showed a dose

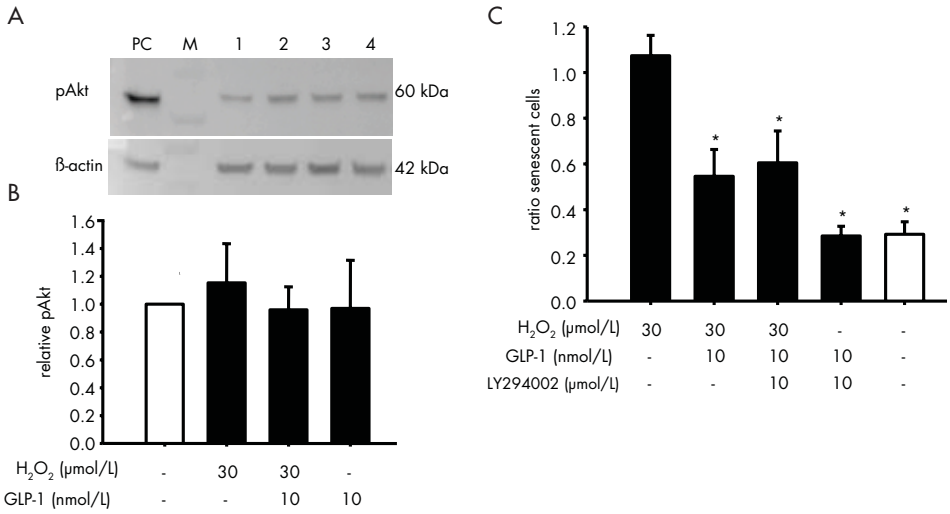

Figure 5. Effect of GLP-1 on Akt and PI3K inhibition. A. Representative blot showing phosphorylated Akt (Ser473) and β-actin levels from the following treatment groups: 1 = control, 2 = 30 µmol/l H_2O_2, 3 = 10 nmol/l GLP-1 + 30 µmol/l H_2O_2, 4 = 10 nmol/l GLP-1, PC = positive control (30 minutes medium with 20% FCS and growth factors), and m = molecular marker lane. Cell extracts were prepared after 1 hour H_2O_2 exposure. B. Relative levels of phosphorylated Akt, after GLP-1 or H_2O_2 treatment or a combination of the two. Data from 3 experiments. C. Effect of the PI3K inhibitor LY294002 on the protective effect of GLP-1. Data were obtained from 4 experiments. Open bar represents the non-treated control group. *P < 0.05 compared with H_2O_2 groups.

response effect on the cellular senescence in combination with GLP-1, with a complete abolishment of the GLP-1 protective effect at a concentration of 1 µmol/L H89 (Figure 6B). Similar results were obtained with another PKA inhibitor KT 5720 (Supplemental Figure IV B). These results indicate that PKA activation by GLP-1 is sufficient to prevent cellular senescence in HUVECs under these conditions.

GLP-1 activates CREB and induces expression of HO-1 and NQO1

A downstream target of PKA is the transcription factor CREB, which is activated by PKA phosphorylation and regulates expression of numerous genes in conjunction with other transcription factors. Western blot analysis using an anti phosphorylated CREB antibody showed that GLP-1 treatment increased relative phosphorylated CREB levels by 52% as compared with the control (Figure 6C and 6D). This effect was abolished by PKA inhibition using H89 (Figure 6C-D). Similar results were obtained with forskolin (Figure 6C and 6D). Quantitative PCR analysis of heme-oxygenase 1 (*HO-1*), a reported anti-oxidative target

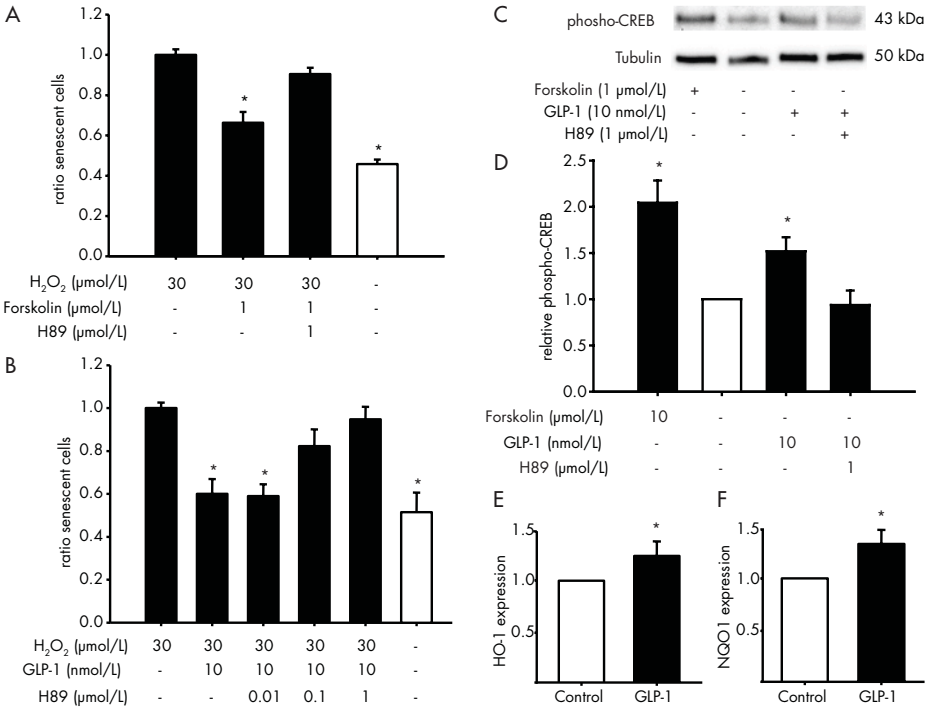

Figure 6. GLP-1 mediated protection is PKA dependent and involves CREB phosphorylation and induction of anti-oxidant genes. A. Effect of forskolin on cellular senescence with or without the PKA inhibitor H89. Open bar represent non-treated control group. Data are expressed as mean ±SE and were obtained from 4 experiments. *P < 0.05 compared to H_2O_2 group. B. Effect of the PKA specific inhibitor H89 on GLP-1 mediated cellular protection. Open bar represents the non-treated control group. Data are expressed as mean ±SE and were obtained from 4 experiments; H89 0.01 µmol/l + 10 nmol/l GLP-1 was repeated 3 times. *P < 0.05 compared with H_2O_2 group. C. Representative blot of phosphorylated CREB protein of HUVEC samples treated with forskolin, GLP-1 or GLP-1 in combination with H89. α-tubulin was used as loading control. D. Quantification of the relative phosphorylated CREB levels. Phosphorylated CREB levels were corrected for the loading control, α-tubulin, and results are from three different experiments (n=3). The control goup was set at one. Open bar represents the non-treated control group. *P < 0.05 compared with the control group. E and F. Relative expression of HO-1 (E) and NQO1 (F) mRNA levels in HUVECs after GLP-1 treatment. Gene expression is relative to non-treated control samples (n=6), which were set to 1.0. Open bar represents the non-treated control group. *P < 0.05 compared with the control group.

gene of CREB[25], showed a concomitant induction after GLP-1 treatment. Also, another anti-oxidative gene, *NQO1* (NAD(P)H dehydrogenase quinone 1), was significantly induced by GLP-1 (Figure 6E and 6F). These data show that GLP-1 can activate the transcription factor CREB and can induce expression of antioxidative genes.

Discussion

GLP-1 analogues and DPP-4 inhibitors are novel therapeutic agents for the treatment of diabetes mellitus. These drugs are primarily designed to improve glycemic control. Interestingly, evidence is accumulating that GLP-1 and its analogues can also protect cardiovascular damage via ancillary pathways.[15, 26] In particular, GLP-1 has been shown to induce vasodilatation and improve endothelial function.[17, 18] In the present study, we showed that GLP-1 can protect endothelial cells against oxidative stress induced cellular senescence.

In ZDF diabetic rats, increased levels of vascular senescence were observed, which is in agreement with a previous study.[10] Treatment with the DPP-4 inhibitor vildagliptin raised GLP-1 levels and attenuated vascular cell senescence in these animals, almost to lean animal levels. Although DPP-4 inhibitors are used to improve glycemic control in diabetic patients, several studies have reported limited effects in glycemic parameters with these overtly diabetic ZDF rats upon DPP-4 inhibition or treatment with GLP-1 analogues.[27-29] In line with this, analysis of blood glucose and insulin levels at the end of our experiment showed only slight, but not significant, changes in the vildagliptin treated animals as compared with the nontreated ZDF animals. However, we did not perform intermediate analysis or glucose tolerance tests and therefore cannot exclude possible changes in glucose handling in these animals upon treatment. Irrespective of this, our results show that DPP-4 inhibition can attenuate vascular senescence *in vivo*, most likely via GLP-1.

Our *in vitro* model system with primary HUVECs supported the observation that GLP-1 could directly attenuate endothelial senescence and provided molecular details. First, we observed that GLP-1 treatment had a protective effect on ROS-induced senescence in HUVECs. Second, this was associated with a reduction in DNA damage, further supporting a protective effect of GLP-1. We cannot rigorously exclude that *in vivo* also other potential DPP-4 targets might have exerted some effects on the vasculature. We note, however, that the only other established clinically relevant DPP-4 substrate is glucose-dependent insulinotropic polypeptide (GIP)[30], but the receptor of this hormone is strongly downregulated in hyperglycemic ZDF rats.[31] Therefore, we strongly believe that the observed *in vivo* effects must be GLP-1 mediated.

It has been shown in pancreatic β-cells that GLP-1 reduced apoptosis, stimulated survival and proliferation, and increased insulin secretion. These effects of GLP-1 have primarily been ascribed to the activation of downstream cAMP/PKA and PI3K/Akt signaling pathways in these cells.[23, 32, 33] Also, the cardioprotective effect of GLP-1 after ischemia/reperfusion could be abolished by inhibiting cAMP, and PI3K[34], indicating that the protective effects of GLP-1 generally involves multiple pathways. Our data indicate that in endothelial cells PI3K/Akt signaling is not activated by GLP-1 and accordingly, inhibition of this pathway

could not abolish the GLP-1 protective effect. The cAMP/PKA pathway, on the other hand, was clearly required for the GLP-1 protective effect in endothelial cells. Thus, it appears that in different cell types, different pathways are involved in GLP-1 pro-survival or anti-senescence mechanisms. Because the GLP-1 receptor directly activates adenylate cyclase via Gαs,[35] it is not surprising that the cAMP/PKA pathway is generally involved in this mechanism. PI3K activation by GLP-1 is indirect, however, involving IRS2 expression or EGFR transactivation[36], and this latter mechanism is apparently not active in endothelial cells.

We also showed here that GLP-1 could induce phosphorylation of the transcription factor CREB, in a PKA-dependent way, in endothelial cells. CREB phosphorylation results in its activation, and previous studies have shown that CREB phosphorylation is associated with cellular survival in pancreatic β-cells.[33, 37] CREB binding site profiling studies have predicted at least 4000 putative target genes, but probably only a few percent are controlled by cAMP, and specific target genes are difficult to define.[38] We investigated the expression of the oxidative stress defense gene *HO-1*, which is at least partially regulated by CREB.[25] We could show here that *HO-1* expression in endothelial cells was significantly induced by GLP-1. This extends a previous observation in which *in vivo* liraglutide treatment resulted in *HO-1* induction and cytoprotection of cardiac tissue.[39] Another oxidative defense gene, *NQO1*, a potential downstream target of the GLP-1 induced the Nrf2/CREB binding protein pathway[39], was also induced in endothelial cells in our study. These results indicate that GLP-1 can activate oxidative stress defensive pathways in endothelial cells.

Recently, it was also shown that GLP-1 can reduce the inflammatory response of endothelial cells upon exposure to advanced glycation end product (AGE).[40] Because AGE formation correlates with ageing and cellular senescence this could be a second path by which GLP-1 protects endothelial cells. Interestingly, the authors showed that GLP-1 attenuated the expression of the AGE receptor RAGE, and this was mimicked by 8-Br-cAMP, indicating involvement of PKA signaling. This suggests that GLP-1 might activate multiple cytoprotective mechanisms and could have wide-ranging cytoprotective effects in cells. Expression profiling and proteomics approaches would be required to further map these global protective changes exerted by GLP-1.

Our data on endothelial senescence provided clear evidence that the GLP-1 protective effect in this setting was conferred by the GLP-1 receptor. HUVECs express the GLP-1 receptor and both exendin-4 and GLP-1 provided protective effects, which were abolished by the antagonist exendin (9-39). To our knowledge this is the first study that links activation of the GLP-1 receptor to a protective effect on cellular senescence. In a recent report, GLP-1 receptor dependent anti-apoptotic effects were described for exendin-4 in mouse cardiomyocytes[41], which is in line with our observed anti-senescence effects in endothelial cells. Surprisingly, however, they did not observe this effect in endothelial

cells, but these cells were treated with very high levels of H_2O_2 (700 μmol/L). Thus, GLP-1 and exendin-4 can apparently prevent endothelial senescence and cardiomyocyte apoptosis, but not apoptosis in endothelial cells. Interestingly, Ban et al[41] did observe anti-apoptotic effects in cardiomyocytes and endothelial cells with the "inactive" cleaved form of GLP-1, GLP-1(9-36), in a GLP-1 receptor independent manner. Whether this form has anti-apoptotic or anti-senescence activity on endothelial cells *in vivo* is, however, not known. Because DPP-4 inhibition only increases the levels of uncleaved GLP-1(7-36), as performed in this study, we have at this stage only evidence that uncleaved GLP-1 can attenuate endothelial senescence.

In aging and in replicative senescence, NO levels tend to deteriorate[42], and NO is an important factor in preventing or delaying the onset of endothelial senescence.[43, 44] We therefore also considered the role of NO. We could not, however, abrogate the GLP-1 induced protection (Supplemental Figure V), by L-NMMA, a NO synthase inhibitor, indicating that NO was not involved. Interestingly, in endothelial cells anti-apoptotic effects by the cleaved GLP-1(9-36) form did appear to be NO mediated, indicating that this follows a different route, as compared with the anti-senescence effects of GLP-1.[41]

In conclusion, we have shown that GLP-1 has a protective effect on endothelial cells via GLP-1 receptor-mediated cAMP-dependent activation of PKA. The latter was both sufficient and essential for the GLP-1 induced effect. This GLP-1 effect on endothelial cells was independent of the PI3K/Akt survival pathway and did not require NO synthesis. These findings provide further evidence for the potentially extraglycemic, pleiotropic, effects of GLP-1 and DPP-4 inhibitors and could serve as a starting point to further investigate the protective mechanisms and to explore GLP-1 treatment options to delay disease and aging related endothelial senescence.

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Supplemental material

Supplemental methods

Reagents

GLP-1 (7-37) (GLP-1), 8-Bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP), N^G-Methyl-L-arginine acetate salt (L-NMMA), H-89 dihydrochloride hydrate (H89), forskolin, LY-294,002 hydrochloride (LY294002), bradykinin, exendin-4, exendin fragment 9-39 (exendin (9-39)), and KT 5720 were all obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The Accu-Check Aviva kit (Roche, Almere, The Netherlands) was used to determine blood glucose levels at sacrifice. Serum DPP-IV activity (Quantizume Assay System, BIOMOL International, Plymouth Meeting, PA, USA), active GLP-1 (Glucagon-Like Peptide-1 (Active) ELISA Kit (Millipore Corporation, Billerica, MA, USA)) and insulin levels (enzymatic assay ALPCO Diagnostics, (Rat) EIA, ALPCO Diagnostics, Salem, NH, USA) were all determined using commercially available kits, according to the manufacturer instructions.

Senescence associated β-galactosidase staining

Cells or tissue were washed in PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes. Subsequently, cells or tissue were washed and incubated for 18 hours at 37°C with sa-β-galactosidase staining solution (150 mmol/l NaCl, 2 mmol/l $MgCl_2$, 5 mmol/l $K_3Fe(CN)_6$, 5 mmol/l $K_2Fe(CN_6)$, 40 mmol/l citric acid / sodium phosphate dibasic at pH 6.0, containing 1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-*D*-galactoside)).

Comet Assay

HUVEC cells were treated as described above and 24 hours after treatment cells were harvested and approximately 700 cells were placed on a CometSlide in LMAgarose (Trevigen, Gaithersburg, MD, USA). Cells were lysed for one hour at 4°C in Lysis Solution, followed by denaturation for 30 minutes in 300 mmol/l alkaline solution, 1 mmol/l EDTA pH>13. Electrophoresis was done in an alkaline electrophoresis solution (200 mmol/l NaOH 1 mmol/l EDTA pH>13). Comet slides were stained with SYBR Green and photos were taken with a 20x objective. Pictures were analyzed with CASP 1.2.2 software.[1] Experiments were repeated three times and in total more than 220 cells were analyzed per treatment group.

Quantitative Real-Time PCR

Total RNA was isolated from cells by Nucleospin II kit (Machery-Nagel, Düren, Germany) and converted to cDNA by QuantiTect Reverse Transcription (Qiagen, Venlo, The Netherlands).

Gene expression was measured with ABsolute QPCR SYBR Green ROX Mix (Abgene, Epsom, United Kingdom) in the presence of 5 ng cDNA and 200 nmol/l forward and reverse primers. Real-Time PCR was conducted on the Biorad CFX384 (Biorad, Veenendaal, The Netherlands) Primers used: HO-1 forward CAACAAAGTGCAAGATTCT, HO-1 reverse TGAGTGTAAGGACCCATC, NQO1 forward AACTTCAATCCCATCATTT, NQO1 reverse TATAAGCCAGAACAGACT, cyclophilin A forward ACTTCACACGCCATAATG, cyclophilin A reverse ACCCGTATGCTTTAGGAT.

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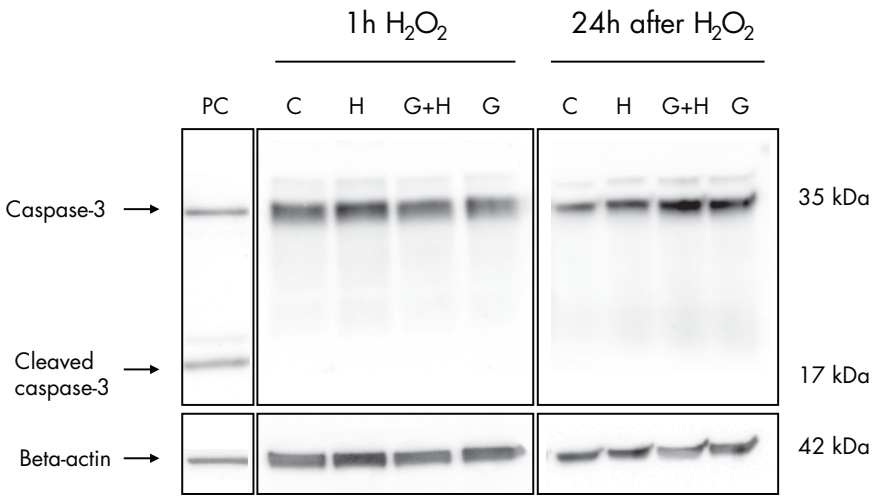
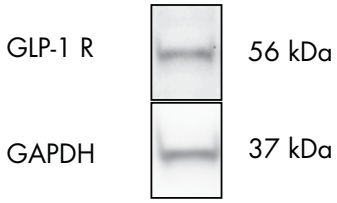
Supplemental table I. Characteristics of lean, ZDF and ZDF + vildagliptin groups at sacrifice. Rats were 25 weeks of age.

	lean	ZDF	ZDF + vildagliptin
Number of animals	*7*	*7*	*7*
Body weight (g)	387 ±11	410 ±19	409 ±8
Plasma glucose (mmol/l)	9.6 ±0.4	23.9 ±1.7*	21.5 ±2.4*
Plasma insulin (ng/10µl)	0.28 ±0.04	0.28 ±0.05	0.34 ±0.04

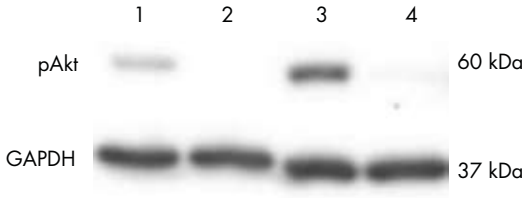
Data presented as means ± SE *P < 0.05 versus lean.



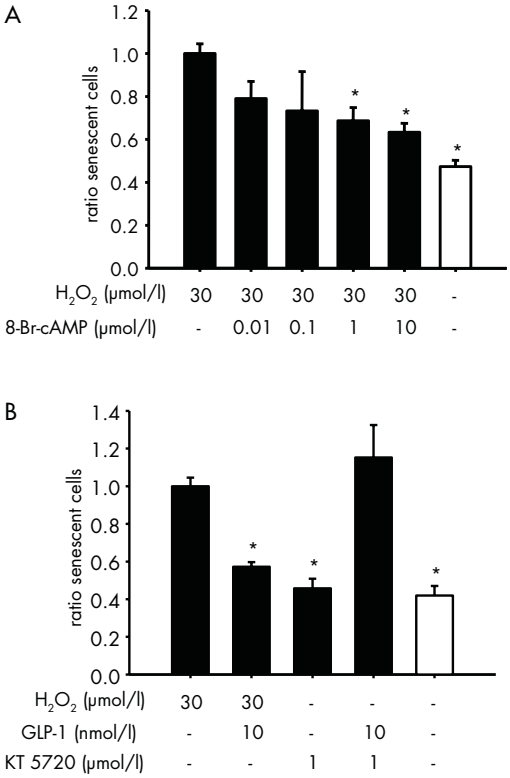
Supplemental Figure I. Caspase-3 cleavage in HUVEC cells treated with 10 nmol/l GLP-1 followed by 1 hour exposure to 30 µmol/l H_2O_2. Caspase-3 cleavage was measured immediatelyafter H_2O_2 (1 hour) or 24 hours after H_2O_2 exposure. C = control group, H = H_2O_2 30 µmol/l group,H+G = GLP-1 10 nmol/l + H_2O_2 30 µmol/l group and G = GLP-1 10 nmol/l group. Positive control (PC) sample is HUVEC cells treated for 24h with 0.5 µmol/l staurosporine. Beta-actin levels detected on the same membrane where used as loading control.



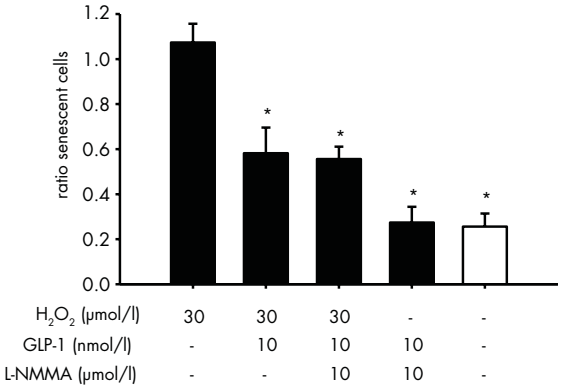
Supplemental Figure II. Detection of the GLP-1 receptor in HUVEC cell extracts. Membrane shows GLP-1 receptor band at 56 kDa, GAPDH (37 kDA) level detectedon the same membrane is shown as loading control.



Supplemental Figure III. LY294002 inhibits phoshorylation of Akt in HUVEC cells. Lane 1: Non-treated control. Lane 2: 30 minutes of LY294002 1 µmol/l treatment. Lane 3: 30 minutes 20% FCS. Lane 4: 30 minutes of 1 µmol/l LY294002 followed by 30 minutes of 20% FCS treatment. Bands represent phoshorylated Akt (60 kDa) and GAPDH levels (37 kDa) on the same membrane as loading control.



Supplemental Figure IV. A. Effect of 8-Br-cAMP on H_2O_2 induced senescence. Data were obtained from 6 experiments. B. Effect of the PKA inhibitor KT 5720 on GLP-1 mediated protective effect. Data from 4 experiments. For both graphs, data are expressed as means ± SE, open bars represents non-treated control groups, *P < 0.05 compared to H_2O_2 group.



Supplemental Figure V. Effect of NO inhibition on GLP-1 mediated cellular protection.
The effect of L-NMMA inhibition on the protective effect of GLP-1. Data are expressed as means ± SE. Data obtained from 5 experiments. Open bar represents non-treated control group. *P < 0.05 compared to H_2O_2 group.

Chapter 6

Summary, discussion and future perspectives

Summary and discussion

As we progress to a society with an increasing average age, the problems that are associated with advanced age become more prominent. Aging is characterized by a slow regression of functionality of tissues and organs to a point that essential functions ultimately fail. The process of aging is often accompanied by the onset of aging-associated diseases. Examples of such diseases are cardiovascular disease, diabetes or cancer that in turn substantially progress the age-related functional deterioration of tissues. On a cellular level, aging is characterized by an accumulation of damage that eventually changes a cell to the extent that it is less capable of performing its primary functions or even looses its function completely. In cell culture, cellular aging is characterized by growth arrest that is accompanied by a prominent phenotype, which is termed cellular senescence. Primary cells that reach this "aging" state attain a growth arrest, but they do not disappear from the culture, in contrast to processes like apoptosis or necrosis.[1, 2] Cellular senescence can occur after cells have reached their maximal replicative potential, also known as the Hayflick limit. However, cellular senescence can also occur before the Hayflick limit is reached due to sub-lethal doses of stress. In senescence research, a distinction is made between the replicative form and the stress-induced form of senescence, and these different causes for senescence may well be amenable to differential therapy. How cells become senescent and the implication of senescent cells in aging tissue have been discussed in chapter 1 of this thesis.

Replicative senescence is associated with telomere shortening. Telomeres consist of repetitive sequences at the chromosome ends and prevent DNA damage checkpoint activation and unwanted recombination.[3] Progressive telomere shortening due to the end-replication problem or DNA damage results in telomere instability, which eventually can induce senescence.[4] Telomerase is the responsible enzyme for telomere elongation, and is primarily active in dividing cells as well as germline cells, stem cells, and haematopoietic precursor cells.[5] Telomerase activity is low in somatic cells[6], but is active in more than 90% of all tumors. Therefore, it has been suggested that increased telomerase activity is required for tumor formation, to overcome cellular senescence.[7] The importance of telomerase activity became clearly evident in telomerase knockout mice, in which telomere length shorten with each generation, accompanied by a premature aging phenotype.[8, 9] In chapters 2 and 3 of this thesis we focused on telomere and telomerase biology. The detrimental effects of telomere shortening on aging, and on aging associated diseases were discussed in chapter 2. In chapter 3 we investigated the effects of voluntary exercise on cardiovascular function in telomerase deficient mice. Exercise is associated with an upregulation of telomerase[10, 11] and is associated with preventing telomere shortening and the effects of aging.[12] Telomerase knockout mice lack the gene encoding the RNA component (TERC) of the enzyme telomerase. The most striking observation was the reduced exercise performance

of the TERC$^{-/-}$ mice in comparison to wild-type (WT) mice. Running wheel performance of TERC$^{-/-}$ generation 3 (G3) mice was less than TERC$^{-/-}$ G2. Telomere length, as expected, was shorter in TERC$^{-/-}$ mice, and G3 had shorter telomeres than G2 mice. Our results were different from results with exercise research in TERT knockout mice. These animals lack the catalytic subunit of telomerase and did not show a difference in exercise performance.[10, 11] The difference in exercise performance was not associated with a strong cardiovascular phenotype in the TERC$^{-/-}$ mice. There was a difference in the maximal vasodilatation of the aorta of the animals. Exercise improved maximal vasodilatation in WT animals, but not in the TERC$^{-/-}$ animals. Strikingly, in a study with aged mice, older animals also performed less in a voluntary wheel running model in comparison to young animals, but exercise improved endothelial function of all animals.[13] Also TERT$^{-/-}$ mice improved endothelial vasodilatation after voluntary exercise.[10] The differences in exercise performance between the TERC$^{-/-}$ and TERT$^{-/-}$ mice are remarkable and at the moment unexplained. Further research into telomerase knockout models might elucidate the fundamental difference in relation to exercise, and might thereby improve our understanding of the relation between telomere length and exercise performance.

In the second part of this thesis we changed from replicative senescence to stress-induced forms of senescence. In particular we studied the effects of stress on endothelial cells. Stress, predominantly oxidative stress, increases with age and the resulting accumulation of damage is an important contributor to aging. For vascular disease, aging is one of the major risk factors[14], and the presence of senescent cells in atherosclerotic plaques are thought to play an important role in atherogenesis.[15] We focused on endothelial cells, as the endothelium is located at the barrier between blood and the underlying tissue, and the endothelium has therefore, a relatively high exposure to physiological and oxidative stress. Endothelial cell senescence is thought to be an important contributor to vascular aging and is suggested to negatively influence endothelial function and might therefore be an important contributor to the onset and progression of endothelial dysfunction.[16, 17] As endothelial dysfunction is present in multiple cardiovascular and other aging associated diseases, improvement of the endothelial function could potentially be an important contributor in ameliorating the detrimental effects of cardiovascular aging.[15, 18] We wondered whether it would be possible to prevent endothelial senescence, and if so, what are the mechanisms involved.

During aging, nitric oxide (NO) levels decrease in endothelial cells and restoring the NO bioavailability could be an interesting option to improve endothelial function.[19] A potent NO stimulatory hormone is bradykinin, and its receptor density decreases with age.[20] We therefore tested the effect of bradykinin on endothelial senescence. In chapter 4, we describe that bradykinin could completely reverse the effects of H_2O_2 induced senescence in an *in vitro* model with bovine aortic endothelial cells (BAECs). Senescence was

induced by sublethal concentrations of H_2O_2. Pharmacological inhibitor studies showed that bradykinin prevented senescence through the G-protein coupled receptor bradykinin 2 receptor (BK2-R). The BK2-R receptor is constitutionally expressed, in contrast to the BK1-R that is only expressed during tissue activation, for instance during inflammation. In our *in vitro* model the protective effect of bradykinin was mediated through the BK2-R, although this does not exclude the BK1-R as a potential contributor to protective effects *in vivo*.[21] Sublethal doses of H_2O_2 resulted in an increase in DNA damage, which would suggest that senescence was induced by the p53/p21 pathway. Protein analysis indicated that p21 levels were upregulated after H_2O_2 exposure, while bradykinin treated cells had p21 protein levels that were comparable to non-treated cells. Further analysis indicated that blocking NO generation could totally abolish the bradykinin mediated effect. How NO prevents senescence is still unknown, some studies implicate that NO might upregulate telomerase[19, 22], although the role of telomerase in response to NO is contradicted in another study.[23] NO is a key regulator of endothelial function, and is associated with vasodilatation, inhibition of platelet aggregation, and reducing the upregulation of pro-inflammatory genes. In addition NO can also upregulate genes that are associated with anti-oxidative genes.[24, 25] Later in this discussion we will focus more on the notion that the activation of anti-oxidative response pathways could be important for the protective effect of NO.

From this point forward, we focused on another hormone in relation to preventing stress induced senescence. Oxidative stress and a decline in endothelial functions are important in the progression of diabetes mellitus.[26] A new class of anti-diabetic drugs have the aim to increase the levels of the hormone glucagon-like peptide 1 (GLP-1).[27] This results in improving glycemic control and in addition reduces pancreatic β-cell apoptosis.[28] A safe and relatively easy approach to increase GLP-1 levels is decreasing the levels of dipeptidyl peptidase-4 (DPP-4), which cleaves active GLP-1 resulting in its inactivation.[29] Interestingly, increasing levels of plasma GLP-1 are also associated with improved endothelial function, independent of the effects of GLP-1 on glycemic control.[30] We therefore focused on the effects of GLP-1 on endothelial senescence. Using an *in vivo* diabetic animal model, with increased endothelial senescence, DPP-4 inhibitor treatment resulted in a decrease of vascular senescence. The signaling pathways that are involved in the protective effect of GLP-1 were subsequently studied in an *in vitro* model with human umbilical vascular endothelial cells (HUVECs). GLP-1 mediated protection against oxidative stress induced senescence was mediated through the GLP-1 receptor and the subsequent activation of protein kinase A (PKA). Activated PKA on its turn upregulated CREB modulated transcription, thereby increasing the expression of protective genes like *HO-1* and *NQO1*.

From part II of this thesis, we can conclude that it is possible to prevent endothelial senescence by two different pathways. (Figure 1) Bradykinin increases, through the activation of the G-protein coupled bradykinin B2 receptor, the bioavailability of NO. GLP-1 activates the

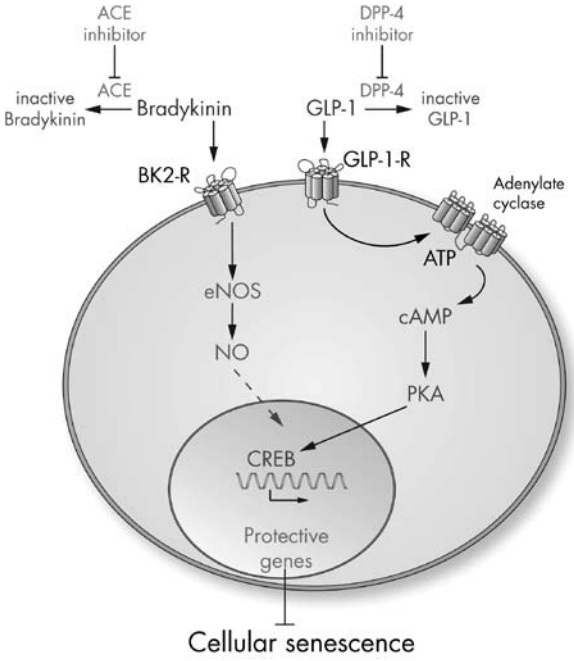


Figure 1. Activation of two G-protein coupled receptors can induce two distinct pathways to prevent cellular senescence.

GLP-1 receptor, which is coupled to another G-protein and activates the transcription factor CREB, resulting in expression of a set of protective genes. It may be argued that both distinct pathways eventually result in the activation of overlapping protective genes or protective pathways. Although in our model the protective effect of GLP-1 was NO independent, a recent study indicates that the GLP-1 analogue, liraglutide, is anti-inflammatory by upregulating intracellular NO.[31] Anti-oxidative stress related genes as, *HO-1* and *NQO1*, are upregulated in response to oxidative stress.[32] They are also upregulated in response to laminar flow (shear stress), and are associated with the protective effects of shear stress.[33] In addition *HO-1* and *NQO1* have been described to be upregulated in response to increased levels of intracellular NO.[24, 25] It seems therefore, that the activation of the bradykinin and GLP-1 receptors could result in an upregulation of similar pathways that are involved in the response to stress. So, the protective effect on a cellular level might be mediated by priming the cells for stress via activation of a similar stress response. Interestingly, this is also suggested for the beneficial effects of exercise. Physical activity increases physiological stress and oxidative stress and therefore activates stress response pathways that might be protective.[34] In that respect, it is interesting to see that activation of stress pathways are also associated with the beneficial effects of caloric restriction. Reducing food intake lowers metabolism, which results in less oxidative stress, but also activates stress response pathways that might be beneficial.[35] Overall this suggests that a limited amount of stress could be beneficial.

Our research into preventing endothelial senescence and the underlying mechanisms was for a large part performed *in vitro*. Therefore, further expansion into animal models and clinical testing will be necessary to test whether the suggested treatments could be beneficial in preventing cellular senescence and improve endothelial function. Our results with the use of the DPP-4 inhibitor, vildagliptin, in ZDF rats showed promising results in preventing senescence *in vivo*. These results have to be extended to other aging animal models, and it would be interesting to research these effects in humans. Interestingly, it has been demonstrated that GLP-1 can improve endothelial function in humans.[30] Increasing NO bioavailability is potentially an important target to prevent endothelial senescence. The *in vitro* results obtained in chapter 4 show that senescence can be decreased by bradykinin, and is likely mediated by increasing NO bioavailability. Angiotensin-converting enzyme (ACE) inhibitors, which are aimed at reducing angiotensin II, also increase bradykinin levels. (Figure 1) ACE inhibitors would therefore, be a logical drug to target senescence. In addition, ACE inhibitors have a beneficial effect on endothelial function by improving intra-cellular NO and eNOS signaling.[36] Of importance for treating senescence by increasing intracellular NO, is to realize that NO is a reactive oxygen species, and therefore could also induce oxidation and cellular damage.[37] In addition, if intra-cellular levels of NO become to high there is the risk of excessive vasodilatation, which could result in hypotension.[38]

Future perspectives

ACE inhibitors, DPP-4 inhibitors, and also GLP-1 analogs, like liraglutide or exanatide, are used in a clinical setting, but so far there has been no research on the effects of these drugs on "aging" of cells in humans. Since these compounds are already investigated in a clinical setting it would be feasible to investigate the effects of these therapies on vascular senescence in humans. At the moment it is unknown how cells would react to combinational therapy, but this might be an interesting future challenge.

Besides bradykinin and GLP-1, other signaling pathways that affect NO levels or activate CREB mediated transcription could be of interest in senescence research. A direct activation of CREB or administration of NO donors could even be a more direct approach to provoke protection. However direct upregulation of NO, which is an oxidative radical, can induce apoptosis[39], and also the direct administration of different cAMP analogues, which upregulates CREB transcription, can potentially induce apoptosis.[40] Most likely the over-activation of these pathways using these methods is detrimental. Therefore, although receptor mediated activation is more pleiotropic, it might be preferred because it gives a more physiological response.

In conclusion, the presence of senescent cells in aging tissue is evident[41-43], but the exact

cause and underlying mechanisms are still unclear. The contribution of stress associated senescence to the aging phenotype is still not completely elucidated. The potential role as a tumor suppressor mechanism might be important against malignant cells.[7] Another aspect might be that in certain cases senescence is preferential over the other forms of cellular decay, like apoptosis, since apoptotic cells disappear from the tissue. For the endothelium it is imaginable that senescence could be preferential over apoptosis.

In cardiovascular diseases and diabetes mellitus, senescence most likely negatively influences the progression of the diseases. Especially, vascular tissue could be sensitive to the development of senescent cells, and be susceptible to the negative aspects of senescent cells.[15] Therefore, preventing senescence could be a potential target to treat aging associated diseases, and targeting endothelial senescence would be an interesting first step. The availability of drugs that can potentially invoke a protective effect on senescence makes it attractive to further study anti-senescence in a clinical setting. Completely preventing aging is probably unlikely, as we cannot avoid cellular damage during life, but improving the response to stress might be within reach. At the moment the best described successful anti-aging therapy is caloric restriction, and although this is simple and very cheap, implementation in our society has proven to be very difficult, as most people are not enthusiastic about dramatically reducing caloric input.

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Nederlandse samenvatting

Onze samenleving wordt gemiddeld steeds ouder, daarmee worden we ook steeds meer geconfronteerd met de nadelige gevolgen van het ouder worden. In de gezondheidszorg is er een toename van ziekten die deels veroorzaakt worden door ouderdom. Voorbeelden van ouderdomsziekten zijn hart- en vaatziekten, diabetes mellitus type II en sommige vormen van kanker. Een belangrijk doel bij de behandeling van patiënten en een doel binnen het huidige medische onderzoek is er op gericht om de nadelige gevolgen van het ouder worden terug te dringen.

Veroudering in organen wordt gekenmerkt door een geleidelijke afname van de functie totdat een orgaan niet meer kan functioneren. De effecten van veroudering zijn daarnaast ook zichtbaar op cellulair niveau. De meeste cellen in ons lichaam ondergaan zeer karakteristieke veranderingen op zowel morfologisch als op intracellulair niveau wanneer de cel ouder wordt. Dit verouderingsproces van cellen wordt in **hoofdstuk 1** besproken. In dit hoofdstuk beschrijven we het ontstaan en de gevolgen van cellulaire veroudering, een proces dat ook wel cellulaire senescence wordt genoemd.

Hoe senescence precies ontstaat, is nog niet duidelijk, maar een belangrijke rol is weggelegd voor eiwitten die betrokken zijn bij de celdeling, de transcriptie van genen en de inductie van geprogrammeerde celdood (apoptose). Hoewel de biologische functie van senescence nog niet bekend is, wordt het steeds duidelijker dat het aantal senescent cellen toeneemt naar mate we ouder worden en dat de aanwezigheid van senescent cellen in verschillende organen zoals in het vaatsysteem of in de nieren groter is bij patiënten die ouderdomsgerelateerde ziekten hebben. Eén van de hypotheses is dat senescence een anti-tumor mechanisme is, dat voorkomt dat een niet goed functionerende cel oneindig kan delen en een kankercel wordt. Dit houdt in dat senescence een intracellulair "failsafe" mechanisme is dat moet voorkomen dat beschadigde cellen zich kunnen verspreiden. Er zijn twee vormen van senescence. De eerste vorm is de replicatieve senescence waarbij een cel senescent wordt na een bepaald aantal delingen. Bij deze vorm van senescence spelen de uiteinden van de chromosomen (de telomeren) een belangrijke rol. De rol van de telomeren wordt besproken in de hoofdstukken 2 en 3. Daarnaast kan senescence ook geïnduceerd worden in cellen die nog niet hun maximale aantal delingen hebben gehaald, op het moment dat ze worden blootgesteld aan verschillende vormen van stress, zoals vrije zuurstof radicalen, radioactieve straling of UV straling. Deze tweede vorm van senescence wordt ook wel stress geïnduceerde senescence genoemd. In hoofdstuk 4 en 5 ligt de nadruk meer op deze vorm van senescence.

Bij het ontstaan van replicatieve senescence spelen de telomeren een belangrijke rol. In **hoofdstuk 2** bespreken we de effecten van telomeerverkorting op het ontstaan van senescence en veroudering. De telomeren bevinden zich aan de uiteinden van de chromosomen, waar geen functionele genen aanwezig zijn en bestaan uit een opeenvolging van identieke stukjes DNA. Door de repeterende structuur kunnen de telomeren een cap structuur aannemen, die

ervoor zorgt dat het uiteinde van het chromosoom beschermd blijft. Bij te korte telomeren wordt deze cap niet meer gevormd en zullen de uiteinden van de chromosomen worden herkend als gebroken DNA, waardoor DNA-reparatie processen worden geïnitieerd, die uiteindelijk kunnen resulteren in senescence.

Uit onderzoek is gebleken dat telomeren korter worden naarmate we ouder worden. De telomeerlengte kan echter sterk variëren tussen de verschillende chromosomen in een cel en metingen bij mensen hebben laten zien dat telomeerlengte tussen personen en bevolkingsgroepen eveneens sterk kan verschillen. Het is daardoor moeilijk om vast te stellen wanneer de telomeerlengte te kort is. Wel is gebleken dat de gemiddelde telomeerlengte van een persoon geassocieerd is met ouderdomsziekten. Telomeren worden korter doordat bij de celdeling de uiteinden van onze chromosomen niet volledig gerepliceerd kunnen worden. Daarnaast zijn telomeren gevoelig voor DNA schade, bijvoorbeeld door verhoogde niveaus van oxidatieve stress wat kan leiden tot telomeer instabiliteit en de inductie van senescence of apoptose. Om te voorkomen dat de telomeren te kort worden in stamcellen en cellen die verantwoordelijk zijn voor de voortplanting, hebben deze cellen een hoge activiteit van het enzym telomerase. Dit enzym is in staat is de telomeren te verlengen. De belangrijke rol die telomerase vervult, is zichtbaar in telomerase knock-out muizen. Deze dieren hebben door de afwezigheid van telomerase activiteit per generatie cumulatieve telomeerverkorting en een versneld verouderend fenotype, wat eveneens sterker wordt in de latere generaties dan in de eerste generatie van deze muizen.

Recent onderzoek heeft aangetoond dat er wellicht een link is tussen telomeerlengte en de gunstige effecten van regelmatige beweging. Daarom hebben we in **hoofdstuk 3** gekeken naar de effecten van vrijwillige beweging op telomerase knock-out muizen. Het belangrijkste resultaat in dit hoofdstuk was dat de telomerase knock-out muizen minder rennen ten opzichte van wild-type dieren. Hoewel we vergeleken met wild-type dieren kleine verschillen in de hart- en endotheelfunctie van de telomerase knock-out dieren zagen, konden deze verschillen niet de verschillen in prestatie in het loopwieltje verklaren. Waarschijnlijk zijn er meerdere kleine verschillen tussen telomerase knock-out en wild-type dieren die het verschil in loopactiviteit veroorzaken, hoewel we op dit moment nog niet kunnen uitsluiten dat er andere grote afwijkingen zijn in organen waar we niet naar gekeken hebben. Daarom zou het interessant zijn om bij vervolg onderzoek ook goed te kijken naar metabolische en neurologische verschillen in combinatie met cardiovasculaire parameters.

In de eerste 3 hoofdstukken van dit proefschrift ligt de focus voornamelijk op het ontstaan van senescence en de rol van de telomeren en het enzym telomerase. In het tweede deel verschuift de focus naar stress geïnduceerde senescence in endotheel cellen en het gebruik van verschillende hormonen om senescence tegen te gaan. In **hoofdstuk 4** beschrijven we in een celkweek model dat het hormoon bradykinine een beschermend effect heeft tegen

het ontstaan van senescence in endotheel cellen. Bradykinine is één van de stoffen die sterk verhoogd aanwezig is bij het gebruik van ACE inhibitors, medicijnen die de bloeddruk verlagen. In een model met bovine aortic endothelial cells (BAECs) hebben we laten zien dat een milde blootstelling aan oxidatieve stress resulteert in senescence, doordat de oxidatieve stress het DNA beschadigt. Het ontstaan van senescence na blootstelling aan oxidatieve stress is door middel van een specifieke kleuring zichtbaar gemaakt. Daarnaast werd senescence aangetoond door de upregulatie van het senescence eiwit p21. Bradykinine behandeling voorafgaand aan de blootstelling aan oxidatieve stress resulteerde in minder DNA schade, leidde tot een afname van de p21 eiwit levels en herstelde de migratie capaciteit van de cellen. Met inhibitor proeven hebben we vastgesteld dat het beschermende effect van bradykinine afhankelijk is van de bradykinine-2 receptor en stikstofmonoxide (NO), een essentiële signaalstof voor endotheelcellen met intracellulaire en extracellulaire functies.

In **hoofdstuk 5** laten we vervolgens zien dat vasculaire senescence ook via een andere moleculaire route kan worden voorkomen. Een relatief nieuw doel in de strijd tegen diabetes mellitus type II is het remmen van dipeptidylpeptidase 4 (DPP-4), door bijvoorbeeld het medicijn vildagliptin. Primair zijn DPP-4 inhibitors erop gericht de insulineafgifte te verhogen en glucagonconcentraties te verlagen in patiënten, door het verhogen van de plasmaconcentraties van het hormoon Glucagon Like Peptide 1 (GLP-1). Dit hormoon heeft naast het effect op het insuline- en glucagonmetabolisme ook een effect op de vaatfunctie. Een opvallend kenmerk van vaten uit diabetes type II patiënten of bij diabetes proefdiermodellen is de aanwezigheid van senescent cellen in de vaatwand. In hoofdstuk 5 laten we in een proefdiermodel voor diabetes zien dat vildagliptin behandeling resulteert in een verminderde hoeveelheid senescence positieve cellen in de vaatwand. Om meer inzicht te krijgen in de onderliggende moleculaire routes hebben we de betrokken moleculaire routes onderzocht in een celkweekmodel met humane endotheelcellen. De cellen werden blootgesteld aan oxidatieve stress, wat resulteerde in stress-geïnduceerde senescence. Behandeling van de cellen met GLP-1 beschermde de cellen tegen stress geïnduceerde senescence. Door gebruik te maken van inhibitors konden we vaststellen dat het beschermende effect gemedieerd wordt door de GLP-1 receptor en dat deze receptor de second-messenger cyclisch adenosine monofosfaat (cAMP) activeert. Het beschermende effect is protein kinase A (PKA) afhankelijk en loopt via de activatie van de transcriptiefactor cAMP response element-binding (CREB). CREB is op zijn beurt in staat om beschermende genen tegen oxidatieve stress te activeren. Opvallend is dat dit beschermende effect van GLP-1 niet afhankelijk is van stikstofmonoxide, zoals het geval was in hoofdstuk 4. Uit hoofdstukken 4 en 5 kan geconcludeerd worden dat het mogelijk is om senescence te voorkomen en dat dit kan via de activatie van twee verschillende moleculaire pathways.

In **hoofdstuk 6** zijn de gevonden resultaten samengevat en besproken in relatie tot de verouderingsbiologie. Interessant is dat de moleculaire pathways die beschermende

effecten hebben ten opzichte van senescence eveneens een rol spelen bij de natuurlijke respons op stress. Voor toekomstig onderzoek is het daarom interessant om te kijken naar de verdere mogelijkheden om deze routes te gebruiken om ouderdomsziekten uit te stellen of tegen te gaan. Daarnaast is het interessant om te onderzoeken in hoeverre de routes nog intact zijn bij oudere patiënten, om een beter beeld te krijgen van de mogelijkheden om senescence tegen te gaan.

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